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Development of Tissue Engineered Vascular Grafts Resistant to Diabetes Mellitus

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DEVELOPMENT OF TISSUE ENGINEERED VASCULAR GRAFTS RESISTANT TO DIABETES MELLITUS

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by
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ABSTRACT

Diabetes Mellitus, characterized by high levels of blood sugar, is a significant risk factor for cardiovascular disease. Almost 25 million Americans are currently diagnosed with diabetes, with numbers rising rapidly. Hyperglycemia and dyslipidemia coexist in diabetes and result in inflammation, stiffening and degeneration in blood vessels which contributes to the development of atherosclerosis and arterial diseases such as coronary and peripheral artery disease. The diseased arteries can be replaced with synthetic materials, but the small-diameter arterial replacements (< 6 mm diameter) fail after 5-10 years because they are not resistant to the damage associated with diabetes. Tissue engineering offers a promising approach to develop decellularized extracellular matrix-based vascular scaffolds, repopulated with the patients' own stem cells, to provide a non-immunogenic, patient-tailored vascular graft with improved remodeling and integration with native tissue. However, the presence of diabetes will structurally modify the implanted tissue engineered grafts. Therefore, it is necessary to develop tissue engineered vascular constructs capable of resisting diabetes-related alterations. The primary focus of this research was threefold: 1) Develop decellularized vascular scaffolds derived from porcine renal arteries with matrix integrity and mechanical properties similar to native blood vessels, 2) test the resistance of scaffolds treated with penta-galloyl glucose (PGG) to diabetes in functional small animal model, and 3) determine the immunomodulatory effect of adipose derived stem cell differentiation and seeding on scaffold remodeling in a diabetic environment.

First, a complete decellularization procedure of porcine renal arteries was established and optimized to remove all cellular and nuclear material from the scaffolds while still preserving the extracellular matrix components and mechanical properties. Treatment with PGG stabilized the matrix proteins and provided antioxidative properties.

An *in* vivo rat study was conducted to evaluate the scaffolds' biocompatibility and ability to resist diabetes-related inflammation and glycoxidation. In comparing PGG-treated and non-treated groups, PGG-treatment consistently showed increased resistance to oxidative stress, inhibition of inflammatory cells and no limitations towards cell infiltration.

Lastly, adipose derived stem cells were harvested from the rats and differentiated into vascular cells (endothelial, smooth muscle, fibroblasts) and seeded onto the scaffolds before being implanted autologously. The rat study showed that the cell-seeded scaffolds had higher resistance to the inflammatory effects of diabetes and showed increased polarization of macrophages to the pro-healing M2 phenotype compared to the acellular scaffolds, and no limitations towards host cell infiltration.

The ultimate goal of this research was to develop a viable ECM based vascular graft that could survive long-term in a diabetic environment. It is expected that the progress made by this project will have a significant impact on those who suffer from diabetes-related vascular disease and complications. This translatable approach towards developing tissue engineered vascular grafts should allow clinicians to take one step closer to adopt this application for treating cardiovascular disease in diabetic patients and increase the success rate of their treatments.

DEDICATION

This work is dedicated to my parents, Subhash and Aparna Dhulekar, and my sister, Simrita. Your love and support have always been and always will be my source of strength in everything I do. Thank you for all your hard work in raising me, teaching me the importance of education and perseverance, and shaping me into the person I am today.

To my grandparents, Saroj Dhulekar, the late Vasant Raghunath Dhulekar, Aruna Vitkar and Dr. Pramod Vitkar. Your kindness, dedication to family, and strength never cease to inspire me. I am so lucky to be your granddaughter and aspire to live my life with the same integrity, greatness and bravery as you all have.

To my Atya, Kaka, Mona Maushi, and Sonu Chacha, for making me feel loved and supported every day, and for reminding me not to take life too seriously.

To all of my friends, and each person I have crossed paths with during these last 5 years. Each and every one of you has touched my life and my story in some way, I thank you for your friendship and for all of the amazing adventures along the way.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

List of Figures (Continued)

Figure Page

CHAPTER ONE

REVIEW OF LITERATURE

1.1 Diabetes Mellitus and its Impact on Public Health

Diabetes Mellitus is a rising global epidemic. According to the International Diabetes Federation, 425 million adults aged 20-70 years have type 2 diabetes and this number is expected to grow to 629 million by 2045.[1,2] Diabetes is a multifaceted disease, influenced by individual genetics and environmental factors such as diet and lifestyle.[3] The prevalence of type 2 diabetes has seen a dramatic increase in China and India, due to rapid changes in nutrition, food habits and physical activities in those populations. Asians may have an increased tendency to diabetes because they have a higher percentage of body fat mass, greater abdominal obesity, and less muscle mass. It is also observed at a slightly higher rate in men than in women.[2] Diabetic individuals have a two to four-fold higher risk of developing vascular diseases than non-diabetic individuals and cardiovascular disease is the major cause of mortality in patients with type 1 and type 2 diabetes.[3,4] About 12 million patients in the US have some form of peripheral artery disease and the American Diabetes Association estimates that 20-50% of them are diabetic.[5] In 2010, diabetes was the seventh leading cause of death in the US as an underlying cause.[6] In the United States, two-fifths of patients with diabetes have poor control of low-density lipoprotein (LDL) cholesterol, one-fifth have poor glycemic control, and one-third have poor blood pressure control. Additionally, the estimated cost of diabetes in the United States was $245 billion in 2012; this includes $176 billion in direct medical costs such as medications, office visits, hospitalizations and emergency

care, and $69 billion in indirect medical costs such as unemployment, absenteeism, and reduced productivity.[7]

1.2 Characterization of Diabetes Mellitus

There are two primary forms of diabetes, Type 1 and Type 2. Other manifestations of the disease can develop during pregnancy and through conditions such as drug or chemical toxicity, genetic disorders, and endocrinopathies. Diabetes is clinically characterized by hyperglycemia due to chronic or relative insulin insufficiency.[8,9] Diabetes is typically diagnosed by fasting plasma glucose (FPG) measurements or an oral glucose tolerance test. In 2009 the American Diabetes Association (ADA), the International Diabetes Federation (IDF) and the European Association for the Study of Diabetes recommended the use of glycosylated hemoglobin A1C (HbA1c) for diagnosis.[7] Type 1 diabetes accounts for less than 10% of cases, typically developed in childhood or early adulthood, and is a result of genetic and environmental factors that lead to an autoimmune response. In this response, the pancreatic β-cells within the islets of Langerhans are destroyed (**Fig. 1.1)**. More than 90% of the diabetic population in the United States has type 2 diabetes, characterized by insulin resistance, hyperinsulinemia, hyperglycemia and dyslipidemia.[4,8] In this form of diabetes, insulin resistance and compensatory hypersecretion of insulin from the pancreatic islets may lead to decline in islet secretory function **(Fig. 1.2)**. The primary deficiency that results from diabetes is impaired glucose metabolism. Insulin targets muscle, liver and adipose tissue for metabolizing glucose and the response of these tissues to insulin determine the circulating concentrations of glucose, fatty acids and

other metabolites. Blood glucose levels are dependent on the balance of hepatic glucose output by the liver, which insulin suppresses, and glucose uptake by muscle, which insulin stimulates. In adipose tissue, insulin promotes the uptake and storage of fatty acids in the form of triglycerides.[10] Without sufficient production and response to insulin, this balance is altered and hyperglycemia develops. Insulin has several cellular effects and regulates not only glucose metabolism but also lipid and protein metabolism. Any defect in maintaining the balance of these metabolic process is viewed as insulin resistance. Insulin action is conducted by insulin binding to a specific transmembrane insulin receptor, which belongs to the family of tyrosine kinase receptors. There are two distinct pathways for insulin signal transmission. One pathway transmits the metabolic effects of insulin via the signaling molecules AKT/PKB, while the other pathway transmits the mitogenic effects via signaling proteins Ras/Raf/MAP kinase. Activation of insulin signal transduction is what allows glucose transport into the cells (**Fig. 1.3)**.[11]

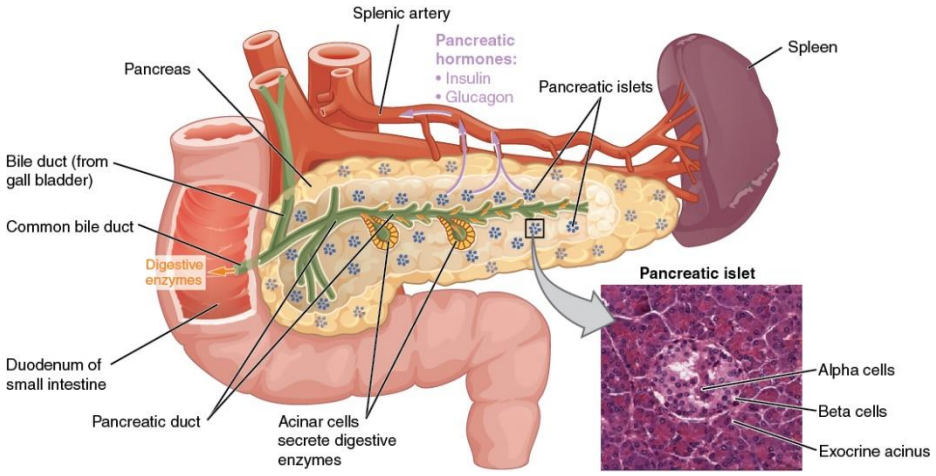

Figure 1.1 **Anatomy of Pancreas (https://opentextbc.ca/anatomyandphysiology/chapter/17-9-the-endocrine-pancreas/)**

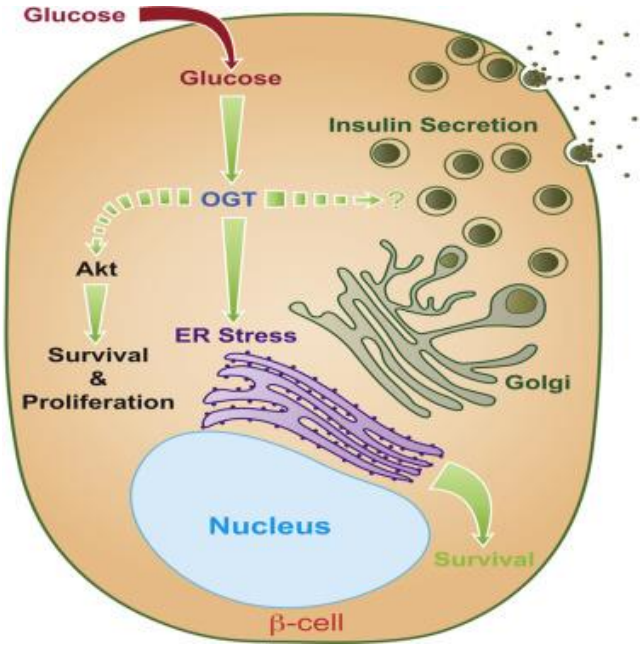

Figure 1.2. Insulin secretion from pancreatic islet beta cell[12]

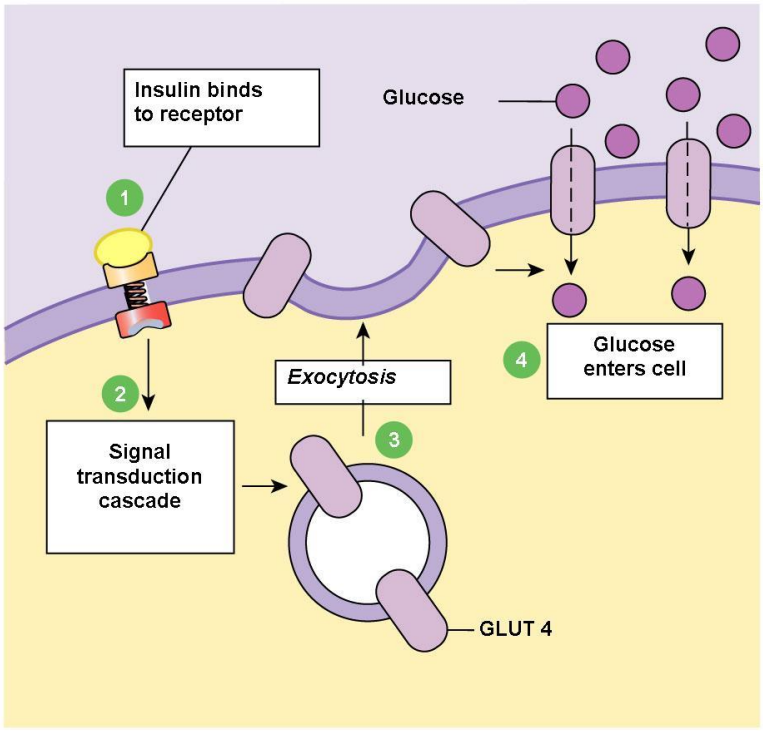

Figure 1.3. Insulin uptake by cell via signal transduction.

(http://www.austincc.edu/apreview/EmphasisItems/Glucose_regulation.html)

1.2.1 Current Treatment Options and Management

Based on our understanding of the behavioral, lifestyle and biological risk factors for diabetes, there are a variety of ways the disease can be prevented and managed. Since adiposity is a significant risk factor, preventing the development of obesity is one of the most important management methods. This includes dietary components such as higher intake of whole grains, green leafy vegetables and nuts, and lower intake of red and processed meat, sugar and moderate intake of alcohol.[2] These dietary changes may allow for improved insulin secretion and/or insulin resistance because of reduced glycemia and lipidemia, reduced ectopic fat, reduced low-grade inflammation, changes in cell

membrane phospholipids, and improvement of intestinal peptide secretion.[13] Physical activity is also recommended because a sedentary lifestyle is associated with increased risk.[2] There is sufficient epidemiologic and experimental evidence that physical activity is involved in the regulation of glucose homeostasis; it has been shown that an increase in walking activity significantly improved 2 hour post-load glucose levels by 23 mg/dL over 12 months in high-risk overweight and obese individuals.[13] Cigarette smoking is also a significant risk factor for developing diabetes and is independent of body weight and other factors.[2]

There are a variety of pharmacologic therapies that can be administered for the prevention or management of diabetes. The American Diabetes Association (ADA) released its Standards of Medical Care in Diabetes for health professionals in 1989. These guidelines provide evidence-based recommendations that are annually updated for the management and diagnosis of diabetes. Metformin is the preferred initial pharmacologic agent for the treatment of type 2 diabetes, if not contraindicated and if tolerated. It is effective, safe, and inexpensive and may reduce the risk for cardiovascular events and death. There is evidence, however, that long-term use of metformin may be associated with biochemical vitamin B_{12} deficiency. Initiation of insulin therapy is recommended in patients with newly diagnosed type 2 diabetes who are symptomatic, have a HbA_{1c} level of 10% or greater, or have a blood glucose level of 300 mg/dL or greater. Generally, a patient-centered approach should be used to guide the choice of pharmacologic agents, which includes assessment of efficacy, hypoglycemia risk, effect on weight, side effects, cost, and patient preferences. After 3 months of therapy

initiation, providers should assess whether the HbA_{1c} target has been achieved; if it has not, therapy should be intensified. Potential combination therapies include a sulfonylurea, thiazolidinedione, dipeptidyl peptidase-4 (DPP-4) inhibitor, sodium-glucose cotransporter-2 (SGLT-2) inhibitor, GLP-1-receptor agonist, or basal insulin. Insulin should be considered as part of any combination regimen for patients with severe hyperglycemia.[14]

1.3 Development of Vascular Complications and Disease in Diabetes

Diabetes is associated with wide-ranging and long-term vascular complications. This is due to chronically elevated blood glucose levels, which damages blood vessels. These complications are grouped as "microvascular" or "macrovascular" disease. Microvascular complications include retinopathy, nephropathy, and neuropathy. The macrovascular complications are arterial diseases that lead to accelerated cardiovascular disease. To put this in perspective, a diabetic individual has a risk of myocardial infarction equivalent to nondiabetic individuals who have previously had a myocardial infarction.[15] There are several factors contributing to this; diabetes causes accelerated atherosclerosis accompanied by higher inflammatory infiltrate (macrophages and T cells), larger necrotic core size, and more diffuse atherosclerosis in the coronary arteries[15,16] (**Fig. 1.4)**.

Clinical studies conducted by DeBakey and Glaeser showed that the progression of atherosclerosis lesions in response to systemic risk factors differed in 4 distinct vascular regions: coronary arteries, branches of the aorta, abdominal visceral arteries, and the terminal abdominal aorta.[17] Hyperglycemia activates nuclear factor-κB, which

regulates several pro-inflammatory and pro-atherosclerotic target genes in endothelial cells (ECs), vascular smooth muscle cells (SMCs), and macrophages. Furthermore, hyperglycemia stimulates oxidative stress, which is another major contributor to developing atherosclerosis.[18] Many of the factors that are related to vascular disease are influenced by endothelial dysfunction caused by hyperglycemia. These include, LDL oxidation, decreased endothelial nitric oxide (NO) production, and increased circulating levels of ICAM-1, IL-6, IL-18, and TNF-α. These responses can be prevented by antioxidants which supports the theory that oxidative stress has a major pathogenic role in diabetes complications.[3] As a result of high glucose concentration, EC quiescence is lost, the cells acquire new phenotypes, and their normal function is impaired, initiating endothelial dysfunction.[19] Endothelial dysfunction is a dominant feature of diabetes and is associated with and predicts cardiovascular disease. This, in combination with the impaired vascular response to insulin resistance results in a variety of pathological conditions.[20] Atherosclerotic cardiovascular disease is the primary cause of death and disability among patients with diabetes, developing approximately 14.6 years earlier and with greater severity than in individuals without diabetes. The high prevalence of coronary and peripheral artery disease in diabetic individuals has been acknowledged for more than a century, yet there has been little improvement in cardiovascular event rates by glucose lowering.[21] In addition to greater atherosclerotic plaque burden, patients with diabetes have higher atheroma volume and smaller coronary artery lumen diameter than people without diabetes.[21,22] In vitro and in vivo models of hyperglycemia have consistently shown a direct effect of hyperglycemia on endothelial dysfunction,

atherosclerotic lesion severity and complexity, and plaque burden.[21,23–25] High glucose concentrations also lead to macrophage proliferation and enhancement of the inflammatory response, and impairment of insulin signaling at various points in the insulin signaling pathway in ECs, VSMCs and macrophages promotes further progression of atherosclerosis, as well as disrupting activation of endothelial NO synthase in ECs.[21,26–28]

Dyslipidemia and diabetes typically occur together, and lipid abnormalities affect 60% to 70% of people with diabetes. LDL cholesterol particles are more atherogenic in diabetes, with denser, small particles that are more prone to modification. Diabetic dyslipidemia is characterized by elevated triglycerides, low high-density lipoprotein (HDL) cholesterol, and higher concentrations of apoB-containing particles. The mechanisms of diabetic dyslipidemia are not entirely understood, but metabolism of very-low-density lipoprotein (VLDL), the main transporter for fasting triglycerides, is insulin-regulated. Insulin suppresses lipolysis and regulates circulating free fatty acids, which are substrates for VLDL cholesterol assembly and secretion. Elevated free fatty acids impair insulin signaling and cause inflammation along with pancreatic β-cell dysfunction. The resulting hyperlipidemia within the atherosclerotic plaque leads to recruitment and migration of monocytes and other immune and inflammatory cells into the vascular subendothelial layer. The recruited macrophages express scavenger receptors that engulf native and oxidized low density lipoprotein, forming foam cells, which increase production of chemokines and cytokines.[21,29]

Another typical characteristic of diabetic vascular disease is calcification, and unfortunately, the mechanisms driving excess calcification in diabetes are not fully understood.[30] Vascular calcification occurs by the deposition of calcium or phosphate crystals in the vascular system.[31] Atherosclerotic lesions in diabetic environments are more likely to be calcified. Diabetic individuals have higher coronary artery calcification than those without diabetes, and calcified plaque burden like that of older individuals without diabetes mellitus. Additionally, people with diabetes are at a higher risk of developing peripheral artery disease with a predisposition for the distal tibial artery, and tibial artery calcification is linked with increased risk of limb amputation and mortality. Hyperglycemia and development of AGEs accelerate calcification, and arterial inflammation associated with diabetes is also linked to increased levels of tumor necrosis factor-alpha, which is a mediator of arterial calcification.[21] There are two primary types of vascular calcifications: medial and intimal;[32] medial vascular calcification is characterized by the deposition of hydroxyapatite and is mostly localized in the peripheral arteries and deposited along the elastic lamina and ECM. This type is typically associated with diabetes and chronic kidney disease (CKD). The consequences for medial vascular calcification are vascular stiffness, impaired hemodynamic regulation, and increased cardiac post-load; it is also a strong predictor of lower extremity amputation and mortality in patients with type II diabetes.[31,33] Intimal vascular calcification is systemic and is activated by oxidative stress and inflammatory pathways. Calcifications can also be classified according to their structure: spotty or granular calcifications are associated with ruptured plaque while diffuse, homogenous, or sheet-

like calcifications are associated with plaques less susceptible to rupture. Acetylated LDL in the presence of dyslipidemia has shown to increase the osteogenic phenotype of cultured VSMCs by 3-fold.[31,34] Inflammation is also a major inducer of vascular calcification; osteogenesis is associated with local inflammation and macrophage infiltration in atherosclerotic plaques of $ApoE^{-/-}$ mice.[35] In the lower limbs, calcification is common in intimal plaques leading to femoral artery occlusion. Hyperglycemia, combined with elastin degradation products increases osteogenic markers such as osteocalcin and Runx2 in vascular cells.[31]

The link between diabetes mellitus and macrovascular disease was made more than 40 years ago.[36] The major consequence of developing diabetes-accelerated atherosclerosis is the development of coronary artery disease (CAD) and peripheral artery disease (PAD). Several epidemiological surveys have shown a 2- to 4- fold increase in the risk of developing PAD in diabetes, determined by an ankle-brachial index of equal to or less than 0.90.[36–38] In the German Epidemiological Trial on Ankle Brachial Index Study, 26.3% of the patients with diabetes developed PAD compared to 15.3% of the non-diabetic patients.[39]

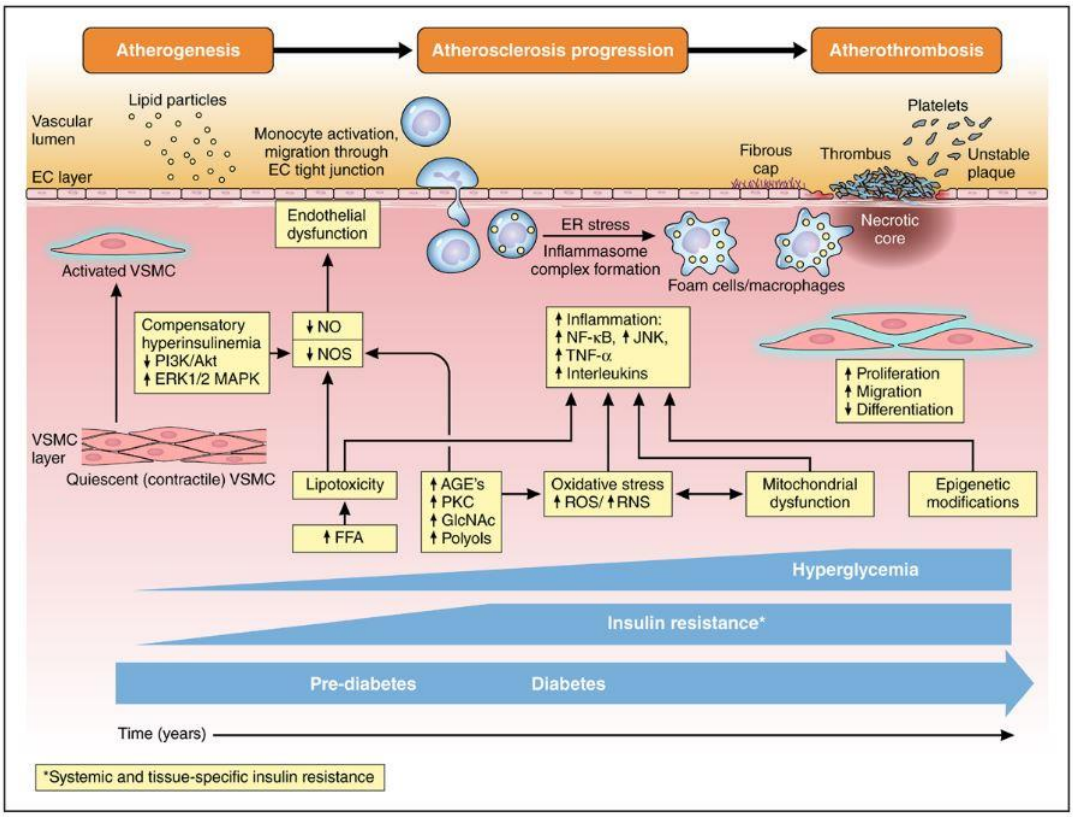

Figure 1.4. Development and progression of atherosclerosis in diabetes mellitus[21]

1.3.1 Effect of diabetes on tissue-specific cells

Many of the initial pathologic responses to glucose are brought about by the vascular cells that directly encounter hyperglycemia.[18] ECs regulate vascular function and structure. The monolayer of ECs is a dynamic and selective barrier that maintains a non-thrombogenic surface, controls transfer of molecules across the vascular wall, and regulates blood flow, immune and inflammatory processes.[40] It is important to note that the intracellular glucose concentration of endothelial cells mimics the extracellular environment.[41] In hyperglycemia, the endothelium loses its non-adhesive property and monocyte adhesion to ECs is one of the initial atherogenic steps. Hyperglycemia and

AGEs also stimulate ECs to produce superoxide ion, contributing to increased oxidative stress. Glucose is believed to stimulate SMC proliferation, migration, and altered reactivity, and activation of matrix-degrading metalloproteinases can lead to plaque rupture and arterial remodeling.[18] Hyperglycemia stimulates the inflammatory "synthetic" phenotype of smooth muscle cells and enhances their migration and proliferation during atherosclerosis. The third type of vascular cell, the adventitial fibroblast, also proliferates and migrates to the media and intima in diabetes, contributing to smooth muscle hypertrophy, neointimal growth, plaque thickening and fibrous cap formation. These fibroblasts also play a role in the arterial response to injury, which stimulates their activation and differentiation into myofibroblasts, the contractile cells involved in wound healing and adventitial remodeling. The adventitia is recognized as the primary site of inflammation in the progression of vascular disease.[5] Hyperglycemia and defective insulin signaling of endothelial cells can promote the adhesion and diapedesis of monocytes, which become activated by circulating chemokines. These recruited monocytes differentiate into macrophages, and express scavenger receptors that engulf both native and oxidized low density lipoprotein to form lipid droplets within their cytoplasm, also known as "foam cells", which consequently increase the inflammatory response through the further release of chemokines and cytokines.[21,42] Monocytes cultured in high glucose concentrations or isolated from diabetic patients are activated and induced various inflammatory mediators such as protein kinase C and nuclear factor κ-B. These factors also promote oxidative stress; similar effects of hyperglycemia have also been observed in T lymphocytes.[18] Adhesion molecules such as P-selectin and

VCAM-1 are upregulated within the vasa vasorum which contributes to the accumulation of inflammatory cells. As matrix components are modified under diabetic conditions, the cells detect and respond to altered matrix stimuli by pathological remodeling and increased stiffness of the vascular wall.[5]

1.3.2 Advanced Glycation End Products (AGEs)

Elevated glucose levels along with excess circulating lipids are responsible for the glycation of proteins, which leads to the formation of advanced glycation end products (AGEs). These are crosslinks formed via oxidation with the proteins of the arterial ECM, collagen and elastin. The presence of these AGEs creates a highly inflammatory and oxidative environment which results in an activated and dysfunctional endothelium, resulting in further acceleration of vascular disease formation.[18,43] Non-enzymatic glycation, discovered by LC Maillard, is defined as the reaction of reducing sugars by amino groups. The initial cascade of chemical reactions leads to formation of intermediate products and then a variety of derivatives called advanced glycation end products (AGEs), this process is known as the Maillard reaction. Glycation occurs spontaneously in a concentration-dependent manner starting with a nucleophilic addition between a free amino group and a carbonyl group from glucose (or any reducing sugar) to form a reversible Schiff base. The Schiff base rearranges into Amadori products which may be fragmented by glycoxidation to form short-chain reactive compounds such as glyoxal (GO) and methylglyoxal (MGO). These reactive compounds react with amino groups of biologic molecules, generating a wide variety of adducts and cross-links called AGEs.[3] Examples of such compounds include (carboxymethyl)lysine (CML),

pentosidine, and pyralline.[44] It is apparent that glycation is a function of both the duration of exposure and concentration of glucose. Protein glycation is a spontaneous reaction and is influenced by several variables such as pH, temperature, degree and duration of hyperglycemia, half-life and concentration of protein and permeability of the tissue to free glucose.[45] AGEs can also be formed through auto-oxidation of glucose (glycoxidation) and oxidative stress.[46]

There are multiple consequences of AGE formation on the ECM. ECM is formed out of proteins with a slow turnover rate, such as collagen, elastin, glycoproteins, and proteoglycans, which maintain the integrity and elasticity of the vessel wall. Metalloproteinases such as collagenases and elastases are produced by vascular cells and macrophages and degrade ECM. The formation of cross-links between glucose or AGEs and ECM proteins decreases the flexibility and permeability of large arteries and leads to a general dysfunction of collagenous tissues in diabetic patients. Glycation and AGE modification of collagens decreases their turnover and result in arterial stiffness and intima-media thickness. Additionally, glycated collagen is associated with endothelial cell senescence and apoptosis. Collagen glycation also increases the adhesiveness of neutrophils and inhibits their chemotaxis and chemokinesis which weakens their host-defense capacity in diabetic patients. Furthermore, glycated collagen results in premature endothelial senescence and apoptosis. AGEs have the ability to modify tissue and cellular proteins and alter their functions.[3] This is due to their ability to trigger proinflammatory, profibrotic and procoagulant cellular responses.[46] MGO and GO can induce the formation of cross-links on tyrosine kinase receptors such as epidermal growth

factor receptor (EGFR) and platelet-derived growth factor-β (PDGFR-β), which impairs their activation by their own ligand and subsequently impairs cell migration, survival and proliferation. MGO also contributes to the pathogenesis of insulin resistance by altering insulin-induced signaling.[3] AGEs are involved in the development of atherosclerosis either by direct tissue deposition and interaction with the receptor for AGEs (RAGE). Tissue deposition of AGEs results in crosslinking the ECM proteins leading to artery stiffness, and the AGE-RAGE interaction alters cellular signaling, gene expression, and enhances expression of proinflammatory molecules.[46]

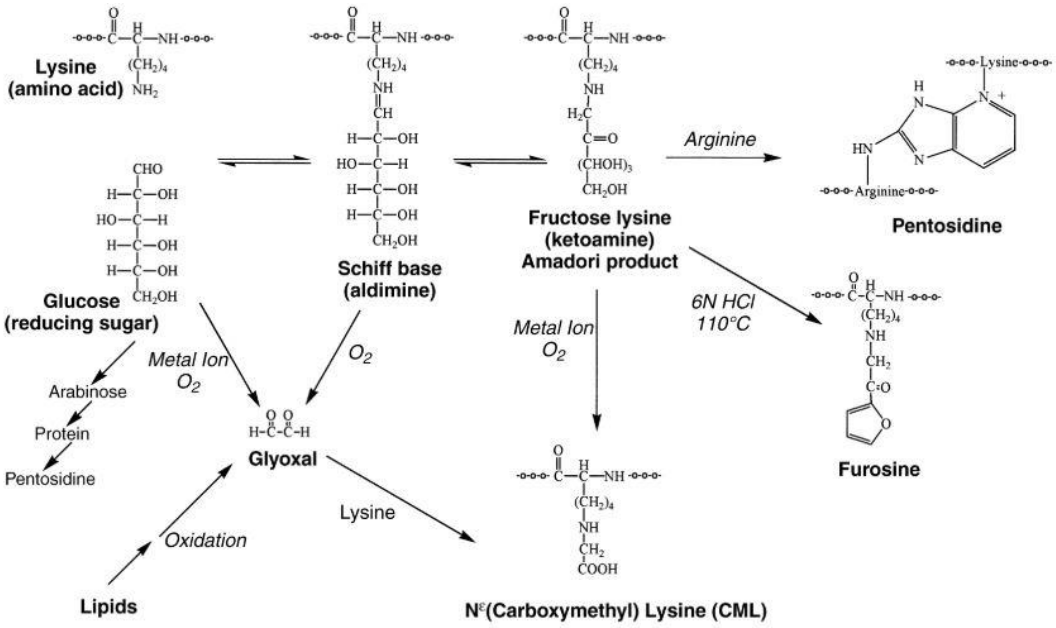

Figure 1.5. Schematic of AGEs formation via the Maillard Reaction and Glycoxidation.[47]

1.3.3 ROS, Oxidative Stress and Receptors for Advanced Glycation End Products

Reactive Oxygen Species (ROS) are normally produced in the body during oxygen metabolism and play an important role in cell signaling. ROS levels are naturally regulated by the biological system's antioxidant ability to neutralize, detoxify, and repair the damage caused to cells.[3] ROS are primarily produced through the electron transport chain in mitochondria, as well as other pathways including xanthine oxidase, lipoxygenase, myeloperoxidase and NO synthase. Oxidative stress results when there is an imbalance between ROS production and the cellular antioxidant defenses. In diabetes, oxidative stress results from an overproduction of ROS, which can occur due to glucose autoxidation, mitochondrial dysfunction, or protein glycation along with decreased antioxidant defenses.[3,21] It has been observed from ROS generation in cultured cells that the increase in ROS production depends on glucose uptake and metabolism, which varies significantly according to the cell type and cell lines. Furthermore, while the high glucose-induced ROS generation may originate from various cellular sources, it is apparent that mitochondria are the initial generator of ROS production. The major sites of physiologic ROS production are complexes I and III of the mitochondrial electron transport chain.[3]

Glycation also affects lipoproteins and the level of glycated LDL and VLDL is significantly increased in diabetic patients and is always higher than the circulating oxidized LDL levels. Glycated LDLs are known to trigger intracellular oxidative stress characterized by ROS production. They also initiate impairment of endothelium-dependent relaxation due to the release of superoxide anion and its condensation with NO

to form peroxinitrite ($ONOO^{--}$). High levels of glycated and AGE-modified LDLs are linked to increased endothelium activation, inflammation and permeability, as well as promoting local oxidative stress and LDL oxidation. These glycated and AGE-modified LDLs are taken up by scavenger receptors which lead to formation of foam cells and atheromatous plaques.[3]

AGEs interact with AGE-specific receptors, which further induce oxidative stress and proinflammatory signaling responses. RAGE are a group of proteins located at the plasma membrane that bind to AGEs. RAGE is the most important system pertaining to AGE binding; its interaction with the ligands initiates proinflammatory signaling, augmenting oxidative stress and NF-κB. RAGE is expressed universally and their expression is upregulated by the ligands in atherosclerotic lesions, glomerular tissues, and retina of diabetic subjects.[3] During atherogenesis, RAGE is highly expressed in activated ECs, SMCs and inflammatory cells.[46] There are many specific cellular effects that occur dependent on AGE/RAGE interactions. These include: reduction of NO synthase (eNOS), endothelium activation resulting from the decrease in NO bioavailability and NF-κB activation that promotes an inflammatory endothelial activation, impaired angiogenesis caused by decreased degradation of AGE-modified ECM components, and stimulation of inflammatory cytokines (TNF-α, IL-1, IL-6), growth factors (TGF-β1, IGF-1), matrix metalloproteinases (MMPs) and chemokines. These agents heighten the proinflammatory response of AGE/RAGE interactions and regulate cell proliferation, hemodynamics, and endothelium permeability. Macrophages from atherosclerotic plaques from diabetic patients show increased expression of RAGE along with

colocalization with inflammatory markers COX-2 and MMP indicates their involvement in plaque destabilization and rupture. Neointimal expansion is also stimulated by AGE/RAGE by activation and proliferation of SMCs. AGE/RAGE interactions also stimulate the biosynthetic activity of endothelial cells by increasing the expression of fibronectin and collagen IV and overall ECM accumulation, resulting in basement membrane thickening and fibrosis.[3] Furthermore, in endothelial cells, AGE binding to RAGE alters the expression of several genes such as thrombomodulin, tissue factor and VCAM-1. This induces procoagulant changes on the endothelial cell surface and increase the adhesion of inflammatory cells to the endothelium. Endothelial AGE-RAGE binding also mediates the increased vascular permeability resulting from diabetes, likely through the induction of VEGF.[48]

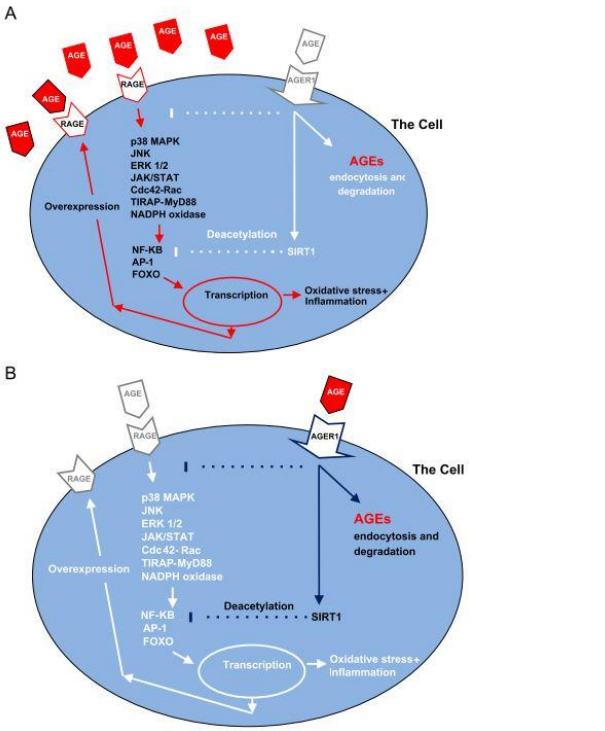

Figure 2: Receptor-dependent pathways activated by AGEs. A substrate excess of AGEs leads to a RAGE overexpression and exacerbation of RAGE-dependent pathways (a), while a low AGEs substrate availability activates mainly AGER1-triggered signals, AGEs endocytosis and degradation (b). Adapted from Poulsen et al., Food and Chemical Toxicology, 2013.

Figure 1.6. Interaction of AGE with RAGE in the cell and the subsequent receptor-dependent pathways activated[49]

1.4 Current Intervention Methods and Diabetes

Over 500,000 arterial bypass operations are conducted every year in the United States; this procedure is needed to restore blood flow downstream due to arterial occlusion, commonly caused by atherosclerosis.[50] Autologous vessels are the preferred source to reconstruct vascular segments with inner diameters smaller than 5 mm, however, close to 40% of patients needing bypass surgery do not have autologous vessels of appropriate quality or length, and even if they are available, problems in *in vivo* remodeling including intimal hyperplasia and mechanical injuries occur frequently.[50,51] Additionally, vascular surgery procedures and revascularization in diabetic patients are

more complicated and likely to have poor outcomes compared with non-diabetic patients undergoing the same procedures. Angioplasty is used to treat arterial stenosis affecting coronary, carotid, renal, and peripheral arteries. This procedure typically sees a vascular response resulting in SMC migration, proliferation, and the secretion of ECM proteins. The incidence of this postangioplasty restenosis is much higher in diabetic patients.[3] Diabetic patients also have a high incidence of restenosis after receiving drug-eluting stents, and coronary artery bypass grafting (CABG) occlusions are also more common at 1-year angiography. Furthermore, while synthetic vascular grafts with medium and large diameters function well, the small-diameter grafts fail rapidly (50% patency rate by two years).[5] Small-diameter blood vessels, which are less than 6 mm in diameter, are located within the peripheral, cardiac and cerebral vasculature. Studies have shown that synthetic grafts placed in these areas experience more rapid thrombus formation and intimal hyperplasia, which greatly limits their use.[52]

Percutaneous coronary or peripheral intervention (PCI/PPI) is particularly challenging in diabetic patients. This is due to the metabolic state and end organ dysfunction. For example, they may have coexisting impairments such as neuropathy and obesity that may negatively impact walking rehabilitation and quality of life in general after intervention. "Metabolic syndrome" is a group of coexisting factors associated with diabetes such as hypertriglyceridemia, decreased HDL cholesterol, hypertension and inflammation that may predispose the patient to poor hemodynamic outcomes after PCI/PPI, including early failure or restenosis. The need for reintervention after PPI presents additional risk of procedural complications including access site

thrombosis or hemorrhage, and lesion thrombosis or embolization.[53,54] Contrast agent-induced nephropathy is also a major concern and limitation as it occurs more frequently in the presence of diabetes. Diabetes is also one of the primary factors in the development of restenosis and total occlusion after plain old balloon angioplasty (POBA), and as a result there is a significantly higher re-intervention rate with frequent referral to CABG.[54] According to the Bypass Angioplasty Revascularization Investigation (BARI) trial, patients with diabetes and multivessel disease who underwent CABG did in fact live longer than patients undergoing balloon angioplasty. This finding led to specific guidelines recommending CABG as the preferred approach for revascularization in diabetic patients.[55] However, even with the moderate success rate of CABG procedures, studies have found that overall CABG occlusions were more common among diabetics at 1-year angiography, especially when saphenous vein grafts were used.[56] Despite advancements and improvements revascularization procedures, diabetic patients still suffer from high incidence of arterial diseases-related mortality which makes it evident that there is a significant need for developing vascular grafts with long-term stability and patency for these patients.[5]

Table 2. Clinical Outcomes and Overall Study Graft Occlusions, Diabetics versus Nondiabetics (N=440)

	Diabetics (n=115)	Nondiabetics (n=325)	*P* Value
Clinical outcomes			
Death at 1 year	0 (0%)	0 (0%)	1.0
Nonfatal myocardial infarction	6 (5%)	42 (12%)	0.02
Repeat revascularization	1 (0.9%)	3 (0.9%)	1.0
Angiography, 1 year			
Any study graft occluded, n (%)	33 (14%)	63 (10%)	0.05
Study saphenous vein graft occluded	22 (19%)	38 (12%)	0.04
Study radial artery graft occluded	11 (10%)	25 (8%)	0.5

Figure 1.7. 1-year angiography shows higher percentage of saphenous vein graft and radial artery graft occlusion in diabetics vs nondiabetics.[56]

1.5 Tissue Engineering Concept and Paradigm

Tissue Engineering is defined as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ."[57] The field of tissue engineering (TE) has great potential for the treatment of cardiovascular diseases, as significant progress has been made in engineering various components including blood vessels, heart valves, and cardiac muscle. The basic goal of cardiovascular TE is to create or regenerate a functional scaffold populated with cells capable of continuously remodeling the ECM.[58] Tissue engineering offers a promising approach to overcome the limitations of synthetic materials by creating bioactive tissue replacements which are completely composed of viable tissue.[51] Synthetic grafts have seen improvements in patency after seeding with autologous endothelial cells onto the lumen of the graft, however, these grafts have not met the performance level of

autologous vessels. Vascular graft failures are most commonly linked to stenosis, thrombosis, intimal hyperplasia, atherosclerosis or infection; atherosclerosis is the main cause of failure after 1 year. Therefore, a tissue-engineered vessel with the ability to grow, remodel, repair and integrate with the native tissue has many advantages and would be very beneficial.[59,60]

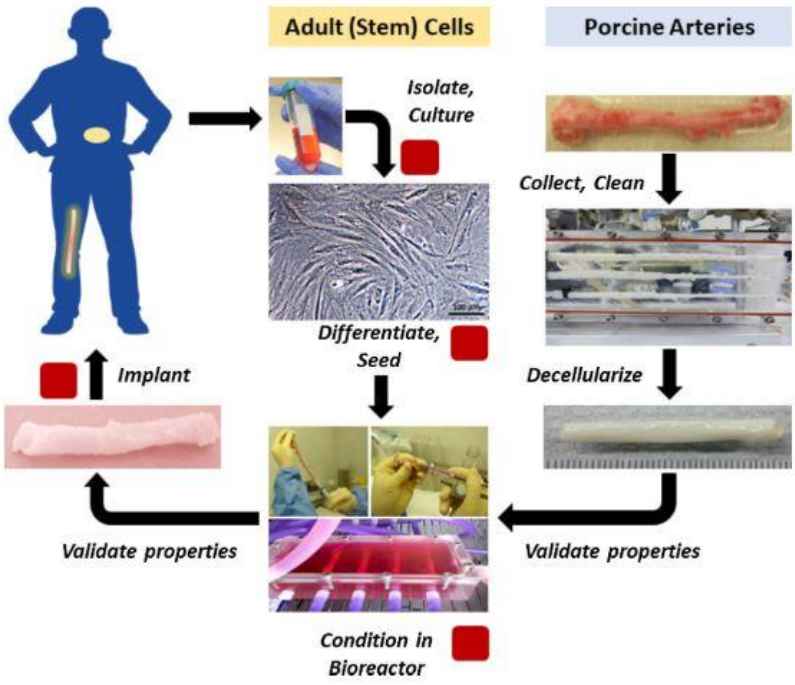

Figure 1.8. ECM based vascular graft tissue engineering paradigm. Stem cells are isolated from the patient, cultured and differentiated. Scaffolds are derived from decellularized blood vessels or other tubular constructs generated in vitro from ECM components, 3D printed scaffolds or cell sheets. The differentiated cells are injected into the scaffolds, dynamically conditioned in a bioreactor and implanted into the patient.[61]

1.5.1 Tissue Engineering Methods

To date, the main reasons for small-diameter vascular graft failure and limitations are due to increased surface thrombogenicity caused by the absence of a functional endothelium and intimal hyperplasia caused by compliance mismatch and chronic inflammation.[51] A TEVG can be constructed using various methods, including cell sheets or seeding cells onto biodegradable or decellularized scaffolds.[57,62] A successful vascular graft should be available off-the shelf, durable under long-term implantation, have low inflammatory response, and should be resistant to thrombosis and infection. The vessel wall should also be elastic and match compliance with the native host vessel.[51] In order to meet these requirements, the focus on tissue engineering methods are gaining increasing interest. When constructing tissue engineered vascular grafts, there are three major components to be considered: a scaffold with the desired shape and mechanical strength, an adhesive matrix, and cells to be incorporated.[63] There are a number of design criteria to be met; since the construct is meant for supporting blood flow, it must withstand the pressures exerted by this flow without bursting or permanent deformation. It should also possess appropriate compliance to prevent high stresses around the anastomosis and have a geometry that will not result in undesirable flow characteristics.[59] Blood vessels demonstrate both viscous and elastic behaviors. At low pressures, elastic fibers and contractile SMCs are responsible for vascular mechanical strength and at high pressures, the recruitment of aligned collagen fibers is responsible for the non-linear stress-strain relationship, ultimate tensile strength (UTS), stiffness and elastic modulus. Therefore, native arteries are compliant and elastic at low pressures and strong at high

pressures.[64] Along with not triggering a negative immune response, the graft should be easily implantable and have the ability to be handled, manipulated and sutured.[59]

Currently, synthetic vascular grafts fabricated from expanded polytetrafluoroethylene (ePTFE), polyethylene terephthalate (Dacron®) and polyurethane are used, however, due to thrombus formation and compliance mismatch, none of these materials have been suitable for producing grafts less than 6 mm in diameter. For example, only 45% of standard ePTFE grafts are patent as femoropopliteal bypass grafts at 5 years. Designing new vascular replacements is increasingly moving towards mimicking the native arterial wall to maintain normal physiologic responses of the vascular wall and control thrombosis and inflammation. Collagen and elastin, which are responsible for tensile strength and viscoelasticity of the blood vessel are key structural components. Furthermore, the endothelial lining is another important design consideration, as the native endothelium is responsible for maintaining a protective, thromboresistant barrier between the blood and tissue as well as controlling vessel tone, platelet activation and leukocyte adhesion.[65]

The first tissue-engineered blood vessel was developed by Weinberg and Bell in 1986. They cultured bovine endothelial cells, SMCs and fibroblasts in layers of collagen gel supported by a Dacron mesh. Physiological pressures were maintained for only 3-6 weeks, but they could demonstrate the feasibility of a tissue engineered graft with human cells. This achievement pioneered the search for a suitable material for a vascular graft and focused the research on finding appropriate coatings and surface chemical modifications for synthetic materials, biodegradable scaffolds and biopolymers.[65]

Since cells in culture cannot organize themselves into a complex three-dimensional structure, the use of a scaffold to provide a template of the construct is a common approach in tissue engineering. This field has seen scaffold construction using a wide range of synthetic and natural matrices with different manufacturing techniques. A variety of synthetic, natural, and hybrid polymer scaffolds have been developed by a number of researchers.[59] Synthetic materials have been incorporated in vascular graft design primarily due to the ease and flexibility of modifying their mechanical properties. Currently, Dacron is the most commonly used for aortic replacement and occasionally as a conduit for femoropopliteal bypass surgery. Dacron grafts are often crimped longitudinally to increase flexibility, elasticity and kink resistance; however, these properties are usually lost soon after implantation, due to tissue ingrowth. In general, the patency rates of Dacron and ePTFE grafts are similar. Polyurethane is also a viable material for consideration; Nakagawa *et al*. developed a poly(ether)-urethane graft reinforced with knitted polyester fibers, which was found to be more durable than ePTFE. Further work has resulted in developing a poly(carbonate-urea)urethane vascular graft that exhibits a compliance similar to human arteries. [65,66]

The generally poor patency rates of synthetic polymers have motivated further research in developing ways to functionalize the luminal surface of grafts and target tissue regeneration. Coatings, chemical and protein modifications, and endothelial cell seeding on inert materials have been applied to improve reendothelialization and inhibit inflammation. Cell seeding has proven to have limited success due to difficulties in cell sourcing and attachment, and retention during pulsatile flow conditions. Methods to

promote *in situ* regeneration of a functional endothelial lining have also been challenging because of chronic inflammatory and prothrombotic responses to the synthetic polymeric materials.[65]

An alternate method of tissue engineering is the use of biodegradable polymers as scaffolds on which layers of cells are grown. The scaffold degrades and is replaced and remodeled by the extracellular matrix (ECM) secreted by the cells. Polyglycolic acid (PGA)is one such polymer that degrades through the hydrolysis of its ester bonds, and in turn, glycolic acid is metabolized and eliminated as water and carbon dioxide. PGA loses its strength *in vivo* within 4 weeks and is completely absorbed by 6 months. Although this approach has shown promising results, some drawbacks exist. Cell sourcing is a challenge including long culture periods ranging between 2 and 6 months, and the proliferative capacity of cells isolated from elderly patients is limited. While the mechanical strength of the materials is comparable to that of native vessels, but compliance mismatch limits long-term patency.[65] Nieponice et al. formulated a scaffold by combining a porous biodegradable elastomeric polymer, poly(ester urethane)urea (PEUU), with muscle-derived stem cells using their novel rotational vacuum seeding technique resulting in a cellularized tubular construct. Results showed that the cells were able to proliferate and populate the polymer while in dynamic culture, retained their stem cell features, and produced collagen when stimulated with ascorbic acid. While this study showed promising results, one limitation was the lack of developing a functional endothelial layer to prevent acute thrombosis upon implantation.[67]

Row et al. incorporated Small Intestinal Submucosa (SIS) and fibrin to engineer vascular grafts. SIS has shown to maintain a porous scaffold structure that enables cell migration and proliferation. In this study, SIS was turned into cylindrical grafts using fibrin glue. These were implanted into the carotid arteries of sheep and exhibited long-term patency and high levels of host cell infiltration. One-month post-implantation, α-SMA expressing cells were dominantly present indicating vascular contractility. They also found that the presence of donor cells was helpful but not necessary for successful host cell infiltration, remodeling or development of vascular function. However, the study determined that the performance of SIS as a small diameter vascular graft is inadequate, likely due to the lack of a functional endothelium.[68]

Despite all of the advances in tissue engineering incorporating synthetic materials, the limitations for use as small-diameter arterial replacements requires the continued search for optimal methods of engineering materials for this specific application. One approach is the use of decellularized natural matrices; this method takes advantage of the structure and mechanical performance of natural ECM while avoiding any adverse immunological reactions. Decellularized grafts, like synthetic grafts, would be readily available and unlike synthetic grafts, would provide the proper microenvironment for supporting cell infiltration, proliferation and differentiation.[50] Malone et al. and Lalka et al. pioneered the research on tissue decellularization by reporting that implanting cell-free arterial allografts does not result in immunologic alterations. Their research showed that treating the tissues with SDS formed an entirely ECM-based tube with morphologically intact elastin and collagen. The primary take away from these studies was the discovery

that we can focus on reducing allograft/xenograft antigenicity instead of immunosuppressing the host.[69,70] Decellularization is the process of removing antigenic cellular material from the tissue. It can be achieved using a variety of chemical agents such as acids and bases, hypo/hypertonic solutions, detergents and solvents, along with physical methods such as agitation, pressure and abrasion. The native architecture of decellularized tissues along with their diverse structural properties give them many advantages for use as vascular scaffolds. The three-dimensional architecture of the ECM and ECM proteins feature cell-signaling components, which encourage adhesion, migration, proliferation, and differentiation of host cells. They also have nearly ideal biomechanical properties.[51]

1.5.2 TEVG Design Considerations

When developing a method for tissue engineering vascular scaffolds, the preservation of ECM is essential and a priority to maintain the tissue's mechanical properties.[59] The potential drawback when processing the ECM is introducing alterations in the matrix composition which could result in improper integration and remodeling of the decellularized scaffold once implanted in vivo, amongst other complications.[50] The ECM is responsible for maintaining the interaction between the cells and the surrounding microenvironment; the cells secrete ECM components and the ECM proteins regulate cell proliferation and differentiation which determines its tissue morphogenesis and homeostasis in development. Any modifications in matrix proteins resulting from the decellularization techniques may be associated with *in vivo* fibrosis, calcification, poor reendothelialization and eventual failure of the implanted scaffold.[50] The primary

challenges facing researchers when designing TEVG include: selection of the appropriate cell types for seeding onto the scaffold, achieving adequate mechanical properties, understanding the process of remodeling and integration with the host vasculature, and selecting the most suitable animal models for evaluation of the grafts *in vivo*.[59]

The vascular ECM allows for the attachment of resident cells while absorbing and transducing shear and strain forces exerted by blood flow. Endothelial cells produce and attach to a basal lamina and aid in formation of the internal elastic lamina. In atherosclerosis and hypertension, or after disruption of the endothelial layer such as after percutaneous coronary intervention, the tunica intima becomes a thick layer of sparse smooth muscle cells and myofibroblasts. The tunica media is made up of radially-arranged fenestrated sheets (lamellae) rich in elastin, immersed in collagen fibers, thin layers of proteoglycans, and smooth muscle cells. The adventitia layer consists of sparse fibroblasts surrounded by ECM, mainly composed of fibrillar collagens and proteoglycans, as well as vasa vasorum. The adventitia is the primary source of tensile strength in blood vessels.[50] Collagen is the most abundant protein within the ECM, encompassing more than 90% of the dry weight. The mechanical properties of the ECM are mostly dependent on its collagen fiber architecture and kinematics.[71,72] Collagen I and Collagen III are responsible for blood vessels' resistance to mechanical stress. Collagen IV mediates migration and adhesion of endothelial cells, so decellularization protocols, particularly enzymatic ones, should factor in that a degraded Collagen IV will negatively affect the *in vivo* reendothelialization of decellularized grafts.

Reendothelialization is crucial in order to decrease calcification and thrombus formation.[50]

Once implanted, the ECM-based scaffold must be surrounded by healthy tissue and subjected to physiologically appropriate mechanical loads during the remodeling and integration process. The host response typically consists of an immediate infiltration of neutrophils and macrophages, followed by a rapid transition to mononuclear cell infiltrate 4-7 days post-implantation. Chronic inflammation can occur when the ECM scaffold is constructed using methods that significantly alter the native structure and composition of the matrix. Ideally, the remodeling process should culminate in the formation of tissue that is site appropriate, functional, and devoid of any chronic inflammatory reaction.[73]

1.5.3. Antioxidant Treatments to Inhibit TEVG Disease in Diabetes

There is sufficient evidence that hyperglycemia-induced oxidative stress is one of the primary culprits contributing to the development of vascular disease; therefore, an effective strategy might be to reduce oxidative stress and reactive oxygen species (ROS) by treatment with antioxidants. There are a number of known enzymatic and non-enzymatic antioxidant mechanisms that have been studied for this application. Enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase; non-enzymatic antioxidants include vitamins A, C, and E, α-lipoic acid, mixed carotenoids, coenzyme Q10 or ubiquinone, copper and zinc minerals, and cofactors folic acid, uric acid, albumin and B vitamins. Vitamin E has shown to prevent lipid peroxidation in vascular tissue of diabetic rats and reduce the progression of coronary atherosclerosis in diabetic mice.[5,74–76] Vitamin C plays a role in

oxidative/inflammatory processes including scavenging ROS, increasing nitric oxide production in endothelial cells, and protecting cell membranes against lipid peroxidation. Several publications also report on the protection against diabetic complications by plant extracts containing bioflavonoids and polyphenols, such as quercetin and resveratrol.[77] Application of antioxidant treatment to TEVGs is a promising approach to stabilizing and increasing the durability of the scaffolds to improve their success rate.

Penta-galloyl glucose (PGG) is a tannic acid derived polyphenol that has elastin-stabilizing properties that has previously shown to reduce biodegradation and calcification of TEVGs.[78] Tannins are direct antioxidants, and polyphenols are based on gallic acid units bound to a polyol core and exhibit high affinity for proline-rich proteins, particularly collagen and elastin. It is known to have many beneficial properties such as antioxidant, anti-diabetic, and anti-inflammatory. PGG binds to the collagen and elastin in the ECM of tissue scaffolds and can slow down their degradation, which is a desirable characteristic for the design of TEVGs for diabetic patients.[78–80] It has also been shown

that PGG is not toxic at local or systemic levels, and therefore can be used safely in tissue engineering applications.[80]

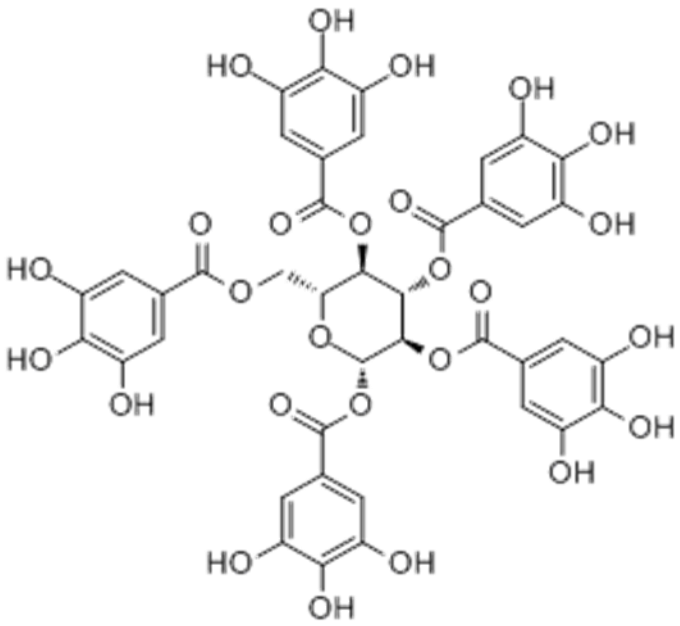

Figure 1.9. Molecular structure of penta-galloyl glucose (www.ichemistry.cn)

1.5.4. Cell Source and Seeding

The type of cells used in the preparation of TEVGs is an extremely important consideration, since the cell type can directly affect the structure of the graft and how it performs *in vivo*. Autologous adult vascular cells such as SMCs, ECs, and fibroblasts have been used in many cases. There are drawbacks to the use of these cells, however, extracting them requires blood vessel biopsies which are very invasive, cause donor site morbidity, and may even be impossible due to impaired vessel quality or availability. Additionally, adult cells in general are limited in replicative and regenerative capacities due to their age.[59] As a result, the use of adult stem cells in tissue engineering and

regenerative medicine has gained a lot of importance, especially due to their potential to improve scaffold remodeling and integration.[81] Stem cells, by definition, are able to self-renew, exist in a undifferentiated state, and are capable of differentiation along multiple lineages.[82] There are several types of stem cells which include progenitor cells, bone marrow mononuclear cells (BM-MNCs), mesenchymal stem cells (MSCs), adipose, muscle-derived stem cells and induced pluripotent stem cells (iPSCs).[59]

Mesenchymal stem cells (MSC), with their multipotent capacity, are the most well characterized source of adult stem cells for application in tissue engineering. These are a heterogeneous population of fibroblast-like cells that are found in most adult organs.[81] MSCs are defined by three main criteria which were established by the International Society for Cellular Therapy: adherence to plastic dishes, specific surface antigens (CD73+, CD90+, CD105+, CD45-, CD34-, CD14 or CD11b-, CD79- OR CD19-, HLA-DR), and in vitro ability to give rise to adipocytes, osteoblasts, and chondrocytes.[83] MSCs can be isolated from a variety of sources including bone marrow, umbilical cord blood, and adipose tissue.[81]

Adipose-derived stem cells are particularly advantages for tissue engineering applications. These cells have been shown to differentiate into both SMCs and ECs and they can be easily harvested in high quantities from adipose tissue aspirate.[59] They have shown the ability to secrete several pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF); and they can differentiate into contractile SMCs with a combination of TGF-β1 and bone morphogenetic protein 4 (BMP-4).[83,84] The most importance characteristics of ASCs for

application in TEVGs include their multipotency, secretory functions and immunomodulatory properties.[83] These cells can maintain their stemness for several passages, and subcutaneous adipose tissue is abundant and easy to harvest. Liposuction is a common surgical procedure that is safe, and a large number of cells can be obtained with minimal risk. Isolation of ASCs can be achieved using a simple procedure in which the adipose tissue is minced and digested in collagenase, followed by centrifugation which results in a cell pellet known as the stromal vascular fraction (SVF). The SVF provides a rich source of adipose derived stem cells (ASCs). Furthermore, repeatable access to subcutaneous adipose tissue offers an obvious benefit over the isolation of MSCs from bone marrow.[81] Approximately 1 in 30 cells from the SVF become the ASC population, with an estimated yield of 100,000 to 1 million ASCs per gram of fat. It has also been determined that gender, BMI, renal failure, diabetes, smoking, cardiovascular disease and peripheral artery disease does not significantly affect ASC yield.[84]

Several studies have demonstrated that cell seeding promotes TEVG patency and longevity, however, the exact mechanism by which the cells promote neo-vessel formation is not entirely known. There is still no known correlation between the number of cells seeded and results in long term graft function. There is are a variety of seeding techniques that have been developed, but many of them are lengthy and limited in clinical applicability. In order to mass produce small-diameter, off-the-counter vascular grafts, it is necessary to develop a cost-effective, reliable, and efficient seeding technique. The most common method used is static seeding, in which a concentrated cell suspension is injected onto a scaffold. There are many limitations to this technique that result in low

seeding efficiency and minimal cell penetration of scaffold walls, however, it is the simplest and fastest method. After application of the cell suspension, the construct is incubated with media to allow cell attachment. Statically seeded cells are incubated with the scaffold from several hours to several days to maximize seeding efficiency. Other seeding techniques include dynamic, magnetic, vacuum, electrostatic and centrifugal as well as incorporation of biological glues such as fibrin or fibronectin.[57] Key features for any seeding techniques include preserving cell viability, providing a uniform cell distribution, attaining high seeding efficiency and minimizing the seeding time.[67]

ASCs are increasingly being studied for their low immunogenicity and immunomodulatory properties including immune stimulation and immunosuppressive effects. ASCs have the ability to reduce inflammation and tissue damage, and also exert effects on both the innate and acquired immune systems with profound implications for monocyte differentiation, macrophage response, and T and B lymphocyte behavior.[85] Suppression of chronic inflammation is key for successful TEVG integration. Stem cell immunomodulation is advantageous for tissue engineered construct integration for reducing inflammatory cytokine production, suppressing cytotoxic T-cell proliferation, and stimulating the secretion of anti-inflammatory cytokines such as IL-4 and IL-10. ASCs may also regulate macrophage polarization by reduction of classically activated pro-inflammatory M1 macrophage phenotype in favor of the alternatively activated pro healing M2 macrophage phenotype.[86]

1.5.5. Mechanical Properties

Blood vessels are load-bearing due to the pressurized fluid flow they support, therefore, the mechanical properties of TEVGs are important design criteria. The graft must have sufficient mechanical strength to maintain scaffold integrity and resist permanent deformation. Graft compliance with the native vessel is also important because compliance mismatch can result in failure and adverse biological responses. Also, the ability of the graft to retain sutures is another important consideration for the surgical methods used for implantation. It has been common to use the gold standard graft, the saphenous vein, as the target for matching mechanical properties; however, patency outcomes may be limited by the same mechanical inadequacies associated with the saphenous vein. The future of vascular tissue engineering may then move towards tailoring specific graft mechanical properties with the intended implantation site. Doing this would require a deeper understanding of vascular biomechanics, interactions between the graft and native vessels, and how to engineer the mechanical properties of a TEVG.[59]

1.5.6. Mechanical Properties of TEVGs after Implantation

Several *in vivo* studies have shown that TEVGs remodel and display altered mechanical properties after implantation. Some cases have shown negative results, with scaffolds becoming weaker or stiffer due to imbalanced ECM deposition, degradation, or calcification. Positive results have also been reported, with grafts becoming more similar to the native tissue. Due to the complex behavior of blood vessels, it is challenging to replicate the mechanical properties of native vessels. Blood vessels are viscoelastic and exhibit a J-shaped stress-strain response. These properties are determined by the proteins

present in the vessel walls: low strains produce small changes in stress due to the compliant and elastic response of elastin fibers; as strains increase, crimped collagen fibers open and engage in a tensile behavior, causing an increase in stress. Elastin plays an important part in the mechanical properties of TEVGS. Elastin fibers are responsible for the elasticity of blood vessels, they prevent dynamic tissue creep by stretching under load and returning to their original shape after the load is removed. This prevents permanent deformation under pulsatile flow.[59]

1.5.7. Remodeling and Integration of TEVGs after Implantation

The exact mechanism by which TEVGs integrate with the host's circulatory system is still unclear. What is known is that the host's immune cells, monocytes, macrophages, and neutrophils, are the major players mediating graft remodeling and neo-tissue formation. Immediately after implantation, monocytes and neutrophils migrate into the anastomosis and an inflammatory response is initiated with debris removal from surgical trauma, via phagocytosis. This process can last several weeks; next, signals are released to direct a shift from inflammation to tissue remodeling and repair. The integration of a TEVG appears to have an atypical response to vascular injury, including intimal thickening and neointima development, along with foreign body reaction, fibrosis, and formation of vascular media.[59]

Monocytes are especially important in early graft remodeling. Monocytes produce cytokines, growth factors, and enzymes important for vascular cell proliferation and tissue remodeling, such as interleukin-6 and -10 and MMPs. SMCs have been found as the major cellular component of the graft's medial layer after 12 weeks. It has been

suggested that host cell invasion of TEVGs occurs at the anastomosis and not through the lumen, and it is possible that the host's immune cells around the anastomosis modulates this process by recruiting circulating progenitors. There is also evidence that seeded cells on TEVGs at the time of implantation are quickly lost or replaced by host cells. It has been shown that ECs seeded onto a decellularized TEVG were replaced by host cells after 30 days in a porcine model. It is still unclear, however, how cells present on TEVGs may interact with the host and influence graft remodeling.[59]

1.5.8. Diabetic Animal models for in vivo studies of TEVGs

A wide variety of animal models have been used to evaluate TEVGs *in vivo*. The primary performance criteria examined are implantability, mechanical performance, biocompatibility, thrombogenicity, and hemodynamics. There is no single model that is optimal for studying all of these, so selection must be made carefully by keeping the specific criteria for study in consideration.[59] Furthermore, the implantation site, vessel diameter and anastomosis are also important considerations when selecting the model. For example, to assess mechanical performance, thrombosis and integration, similarity to the human circulatory system is key; and longer grafts should be selected for longer-term patency assessments.[59,87] Other factors to consider are animal availability, ease of handling, ease of performing the surgery, duration of study, methods of graft analysis post-implantation, and cost.

Rodent models of rats and mice are used extensively in the assessment of TEVGs and are the most widely used preclinical model to study metabolic disorders. In general, they are low cost, easy to care for which allows for increased sample sizes, and are

available with a variety of transgenic strains to examine different molecular mechanisms involved in TEVG remodeling.[59,88] In addition to the species used, there are many other factors that must be considered when selecting the animal model. For example, when determining whether to use male or female rats, in general there is a higher inclination to use male rats. This is because it is widely assumed that the female estrous cycle may induce undesirable experimental variability; additionally, males are also preferred for their more defined disease phenotypes.[88–90] However, diabetes affects both men and women, so it should be taken into consideration that studies should be conducted in both males and females to improve their translational value. Age is another factor that is important to consider; like in humans, ageing affects metabolic parameters in rodents. Age is proposed to be an independent determinant of glucose tolerance, which is known to worsen with age.[88]

There are different ways to induce experimental diabetes mellitus. One way to achieve this is to surgically remove parts of or all of the pancreatic tissue to reduce or fully inhibit insulin production.[88,91] The benefit of this method is eliminating any toxic adverse effects from drugs on other organs. On the other hand, the requirements for specialized training and surgical equipment and the side effects of completely removing pancreatic digestive enzymes and islet hormones is a limitation to using pancreatectomy. Chemical methods such as the use of diabetogenic drugs streptozotocin or alloxan to target the insulin secreting β-cells are also effective approaches. Streptozotocin is preferred over alloxan because it is more stable and less toxic. The use of these drugs essentially creates a stable model of type I diabetes, as they destroy all β-cells, but low

doses elicit only partial β-cell loss which is more representative of type II diabetes. The advantage of this nongenetic model is that the researcher can customize it to simulate the slow pathogenesis of type II diabetes that occurs in humans. The disadvantages of streptozotocin treatment include liver and kidney toxicity and mild carcinogenic adverse effects.[88]

1.6. Conclusions

While progress is being made towards finding a cure and better management methods for diabetes, it is clear that currently patients with diabetes have no diabetes-specific treatment for preventing cardiovascular disease. Tissue engineering for vascular tissues holds immense potential for developing improved re-vascularization methods. However, these methods will be ineffective if they cannot address the complications associated with a diabetic patient. This consideration is extremely important since diabetes is a major risk factor for vascular diseases such as atherosclerosis, peripheral/coronary artery disease, and calcification. The devastating effects of hyperglycemia on blood vessels include extreme levels of inflammation and oxidative stress. Therefore, it should be more than apparent that a tissue engineered construct would experience the same complications once implanted into the patient. While excellent progress is being made in vascular tissue engineering, more research into finding ways to combat the harsh glycoxidative conditions in order to provide patients with diabetes the same chance of treatment success as patients without diabetes.

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CHAPTER TWO

RESEARCH MOTIVATION, SPECIFIC AIMS, AND PROJECT SIGNFICANCE

2.1 Introduction

The potential of Tissue Engineering to develop tissue replacements has offered a great number of opportunities for the treatment of vascular disease. However, one major obstacle that must be overcome is the application of the engineered tissues in patients with **diabetes mellitus (DM)**. This metabolic disease is a major risk factor for cardiovascular diseases and significantly impacts the vascular system. Hyperglycemia, caused by a deficiency in insulin secretion (Type I) or insulin resistance (Type 2), combined with oxidative stress and inflammation leads to vascular damage and disease.[1] Specifically, high levels of glucose non-enzymatically bind to extracellular matrix (ECM) proteins such as collagen and elastin, forming crosslinks that result in the formation of advanced glycation end products (AGEs)[2] and reactive oxygen species (ROS).[3] Revascularization procedures are more complicated in diabetic patients, for example, there is a higher rate of restenosis after stenting, coronary artery bypass grafting occlusions are more common, and many amputation and vascular related deaths are linked with diabetes.[4,5] The need for vascular grafts with long-term viability and patency for diabetic patients is greater than ever. Autologous arteries and veins are the ideal source, however 1/3 of patients do not have vessels suitable for grafting. Furthermore, small-diameter synthetic grafts fail rapidly with only a 50% patency rate by 2 years.[6,7]

Our long-term goal is to develop a biocompatible and ECM-based tissue engineered vascular graft which is capable of inhibiting the effects of diabetes-related

inflammation and glycoxidation. To achieve this goal, we will study the effects of diabetes on porcine renal artery derived scaffolds and the effects of autologous differentiated stem cells on vascular remodeling.

Preliminary studies performed in our lab have demonstrated the protective effects of penta-galloyl glucose (PGG) on treated scaffolds implanted sub-dermally into diabetic rats. Reduced AGE accumulation and inflammatory response was observed.[1] Adipose tissue-derived stem cells (ADSCs) have also been studied for their differentiating ability and seeding potential, and have shown to reduce inflammatory effects when injected into scaffolds and implanted sub-dermally into diabetic rats.

We **hypothesize** that PGG-treatment and seeding differentiated ASCs onto scaffolds will prevent diabetes-related damage such as degradation and calcification and will delay the process of atherosclerosis and vascular disease (VD) in a circulatory model in diabetic rats.

2.2 Specific Aims

Aim 1: Construct decellularized scaffolds derived from porcine renal arteries.

Hypothesis: The decellularization procedure will completely remove cellular and nuclear material while preserving the ECM and mechanical properties of the scaffolds.

Approach: Porcine renal artery segments (1-2 cm long) will be decellularized using NaOH solution followed by treatment with DNase/RNase solution. Scaffold composition and decellularization will be confirmed by histology and DNA quantification. Mechanical properties of scaffolds will be tested.

Aim 2: Test resistance of PGG-treated vascular grafts to diabetes in the circulatory system of small animals

Hypothesis: PGG-treated grafts will resist diabetes-related complications when implanted in a diabetic rat model.

Approach: PGG-treated vascular grafts will be implanted into diabetic rats. The vascular grafts will be monitored for 8 weeks and then explanted to examine patency, thrombogenicity, biochemical alterations, ECM integrity, calcification, inflammatory response, and overall integration with host tissue.

Aim 3: Determine the effect of stem cell differentiation and seeding on scaffold remodeling in a diabetic environment

Hypothesis: Differentiated stem cells seeded on scaffolds will modulate diabetes-related complications and increase host vascular cell infiltration.

Approach: Adipose stem cells (ASCs) will be isolated from rats and differentiated into fibroblasts (FB), smooth muscle cells (SMC) and endothelial cells (EC). The cells will be seeded via injection into scaffolds. The seeded constructs will be autologously placed at the abdominal aorta position as end-to-end anastomoses. After 12 weeks, the constructs will be explanted and evaluated for phenotypic modifications, ECM degradation, glycoxidation products, and host response.

2.3 Significance of Proposed Project

This project is expected to have a significant impact on translational tissue engineering. By implanting these scaffolds in a functional, circulatory rat model, we can observe an authentic physiological response to the engineered scaffolds *in vivo*. The ability

to observe the blood flow, patency, and compliance of the scaffolds provides an increased understanding of the efficacy and applicability of this tissue engineering approach in developing successful vascular grafts. Diabetes is expected to trigger disease and complications in any tissue engineering related products. Therefore, the development of diabetes-resistant tissue engineered vascular construct will allow for autologous blood vessel replacements to improve long-term survival rates and overall contribute to the success of treatment of diabetic patients with vascular disease.

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CHAPTER 3

DEVELOPMENT OF DECELLULARIZED SCAFFOLDS DERIVED FROM PORCINE RENAL ARTERIES

3.1 Introduction

This research focuses on the development of tissue engineered scaffolds using the process of decellularization. The process of removing the cells from the source tissues is one of the first steps in the construction of allogeneic and xenogeneic ECM-based scaffold materials. The effective removal of antigenic epitopes on cell membranes and intracellular components of tissues is vital to preventing an adverse immunological response by the host after implantation.[1] A successful decellularization technique will result in a vascular scaffold with the mechanical properties of native vessels and the immune-privileged characteristics of autologous vessels. This includes physiologically appropriate burst pressure and suture-retention strength. Although there are several ways to decellularize tissues, the optimum method is highly dependent on the type of tissue being decellularized and the mechanical and structural requirements of the scaffold application.[2]

Our goal was to construct non-immunogenic, acellular tissue-engineered artery scaffolds for implantation as vascular grafts in a rat model. We used a NaOH solution and DNase/RNase to decellularize renal arteries derived from porcine kidneys. The use of NaOH as the decellularizing agent has previously been established to effectively decellularize renal arteries while preserving the structure and integrity of the ECM components. The tissue source was selected to obtain the appropriate scaffold dimensions

with the length and diameter similar to the rat abdominal aorta to allow proper compliance and patency. The effectiveness of the decellularization method was determined using histological analysis and quantification of DNA to confirm complete removal of cellular material. The mechanical properties of the decellularized scaffolds were assessed using tensile testing to determine overall strength and suture retention strength, as well as burst pressure testing to ensure the scaffold could withstand physiological blood pressures.

3.2 Materials and Methods

3.2.1. Scaffold Preparation- Kidney Dissection and Renal Artery Isolation

Porcine kidneys were collected from a local abattoir and stored on ice. To isolate the renal artery and its branches from the kidneys, a previously established stepwise dissection protocol was used. First, the extraneous fat was removed, along with the fascia which was pulled away from the kidney. The renal artery trunk was identified and isolated by dissecting away all extraneous fat while protecting the renal artery trunk and its downstream branches.

To dissect the arterial tree from the kidney, small cut on the kidney was made on each side of trunk and thumbs slid through the cuts. Next, the wall of the kidney was gently pried from the renal artery and its branches, carefully to not damage the small arteries by using too much force. Once the kidney was "opened," using blunt dissection, all extraneous tissue was removed from the arterial branches. Gently and carefully, the entire arterial tree was dissected from the kidney, consisting of a hierarchy of branches

ranging from ≥ 3 mm to < 1 mm in diameter. Branches were selected ranging 1-2 mm in diameter and cut so that each segment was 1-2 cm in length and grouped with 5-6 branch segments per group (each group was maintained at approximately the same amount of mass between each group) prior to decellularization. These dimensions were selected to match compliance with the rat abdominal aorta for implantation in future studies.

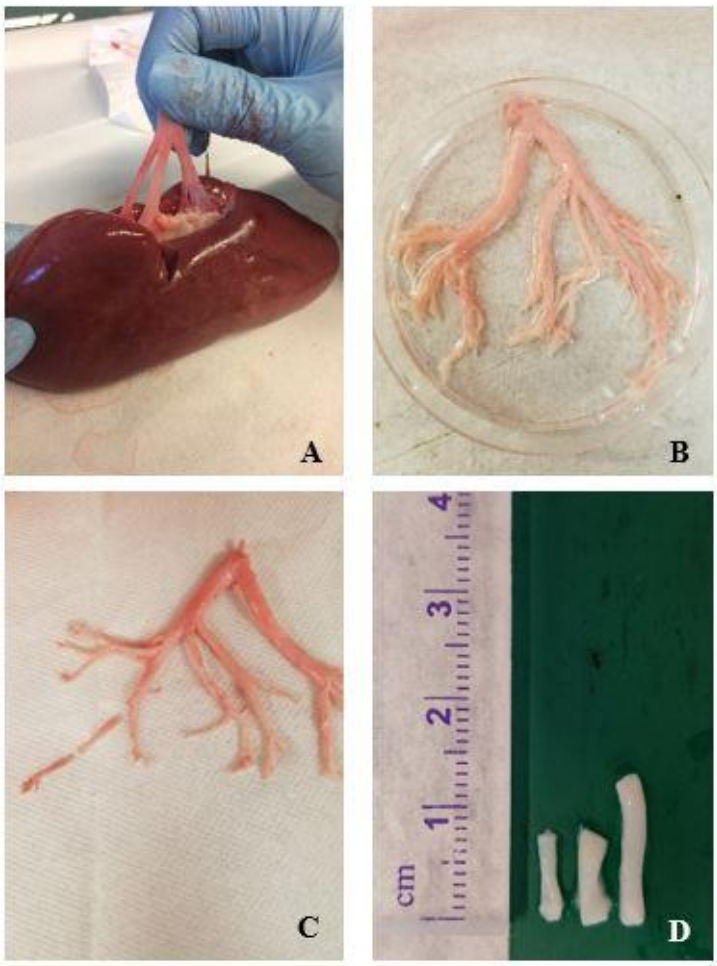


Figure 3.1.1. Dissection of kidneys and isolation of renal arteries. Porcine kidneys are cut to remove exterior fascia and fat; the renal artery trunk is isolated (A). The arterial tree is removed (B) and cut into segments 1-2 cm in length (C, D).

3.2.2. Scaffold Preparation- Decellularization

Following the dissection and isolation, the renal arteries were placed in ddH_2O overnight to induce hypotonic shock to swell and lyse the cells. The next day, each group of arteries were placed in individual 50 mL conical tubes and filled with 45 mL of 0.1M NaOH. The tubes were then placed in a 37℃ shaking water bath for 3 hours. After NaOH treatment, the arteries were removed and placed into new 100 mL sterile specimen cups and filled with ddH_2O. The arteries were rinsed for 5 minutes using an orbital shaker, this step was repeated for 10 rinses. Before decanting the ddH_2O, the pH was checked to ensure the tissues were at neutral pH (~8) and that any residual alkalinity from the NaOH was neutralized. The ddH_2O was decanted and replaced with sterile 1x phosphate buffered saline (PBS) and 1% antibiotic/antimycotic for storage. Effectiveness of the scaffold decellularization procedure was qualitatively assessed using histological DAPI staining (Vector Labs) and Hematoxylin & Eosin staining. Quantitative assessment of DNA content was performed using a Qiagen DNeasy Blood and Tissue Kit (Qiagen cat no. 69504). The decellularized tissue samples and fresh renal artery samples (controls) were frozen in a negative 80℃ freezer and lyophilized prior to following the protocol specified by Qiagen to determine the ng of DNA remaining per mg of tissue. Quantification was performed by absorption analysis using an Epoch plate reader.

3.2.3. Scaffold Preparation – Sterilization

Decellularized scaffolds were sterilized with 0.1% peracetic acid dissolved in 1X PBS and adjusted to pH 7.0 (Fisher Scientific) for 6 hours under agitation at room temperature. Following sterilization, scaffolds were rinsed in ddH_2O 3 times for 15

minutes, followed by two 10-minute 1x PBS rinses. The sterilized scaffolds were stored in a solution of 1x PBS and 1% penicillin-streptomycin-amphotericin (PSA, Corning-Cellgro) at 4°C. All solutions were sterile filtered using a 0.22 um filter prior to use.

3.2.4. Scaffold Preparation – Stabilization (PGG)

Scaffolds were treated with sterile-filtered 0.1% penta-galloyl glucose (PGG, N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium) in isopropanol added to 50mM HEPES buffer in 0.9% saline, at pH 5.5 and room temperature, overnight on an orbital shaker and covered in aluminum foil, as PGG is light-sensitive. After treatment, scaffolds were rinsed three times in sterile PBS for 15 minutes, then incubated in sterile PBS on orbital shaker for 2 hours, followed by three more rinses in sterile PBS for 15 minutes. The PGG-treated scaffolds were stored in 1x PBS and 1% PSA at 4°C.

3.2.5. Scaffold Characterization – Histology

The decellularized and PGG-treated scaffolds were analyzed histologically with H&E (Fisher Scientific) for cell nuclei and ECM morphology, Masson's trichrome (PolyScientific R&D Corp.) for collagen and Verhoeff van Gieson (VVG) stain for elastin (PolyScientific) to qualitatively assess ECM integrity of the acellular and treated scaffolds.

3.2.6. Scaffold Characterization - Mechanical Properties

Uniaxial tensile testing was performed to calculate average young's modulus (E) for the scaffolds to compare strength between fresh, decellularized and PGG-treated scaffolds. The scaffolds were cut 4-6 mm x 10-20 mm rectangular sections and clamped to each end of the MTS test frame (MTS Systems Corp. Eden Prairie, MN). The secured

sections were preloaded to 0.005 N and extended to failure at 2 mm/min using a 10N test frame (MTS Systems Corp).

Suture retention strength was calculated similarly, by cutting scaffolds into rectangular sections and clamping one end to the MTS test frame and placing a single 4-0 braided suture 2 mm from the free edge and tied to the other end of the test frame. The sections were preloaded to 0.005 N and extended to failure at 2 mm/min using a 10N test frame.

Burst pressure was tested using a peristaltic pump to fill arteries with 1x PBS and a pressure transducer (Cole Parmer, Vernon Hills, IL) to record the scaffold's ultimate pressure before rupture. Barbed-Luer adapters were attached to each end of the artery, one end was connected to a port from a master flex rotary pump (Cole-Parmer) connected to a fluid reservoir of 1x PBS and the other end was attached to a 100 psig pressure transducer, USB Data Acquisition System and Personal DAQ view plus software (Omega, Stamford, CT). The pump was run at the maximum speed for 2 minutes while pressure data was collected in mmHg. Maximum pressures recorded were identified as maximum burst pressure values.

3.2.7. Statistical Analysis

Results are expressed as means $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA). Differences between means were determined using the least significant difference (LSD) with an alpha (α) value of 0.05, (n=5).

3.3. Results

3.3.1. Evaluation and Characterization of Decellularized Scaffolds

Initial studies focused on characterization of the decellularized scaffolds before pursuing implantation studies. The porcine renal artery segments were treated in NaOH and DNase/RNase to remove all cells while preserving the ECM methods. Following this process, scaffold color and appearance was indicative of effective removal of cellular material. Before decellularization, the arteries were pink and red in color, but after the decellularization procedure they became progressively paler, eventually turning white. As seen in **Fig 3.1.2**, histological analysis of the decellularized scaffolds showed complete elimination of cells (H&E and DAPI staining). VVG Trichrome staining showed overall complete preservation of elastin fibers, as well as "pores" where the original cells used to be. Collagen fibers were not visibly apparent, however, since the goal was to construct a primarily elastin-based scaffold, this was not a major component for characterization. To quantitatively validate the completeness of the decellularization procedure, DNA quantification analysis was performed (Qiagen DNeasy Blood and Tissue Kit) and showed a 95.6% reduction of DNA from the arteries (**Fig. 3.1.3)**.

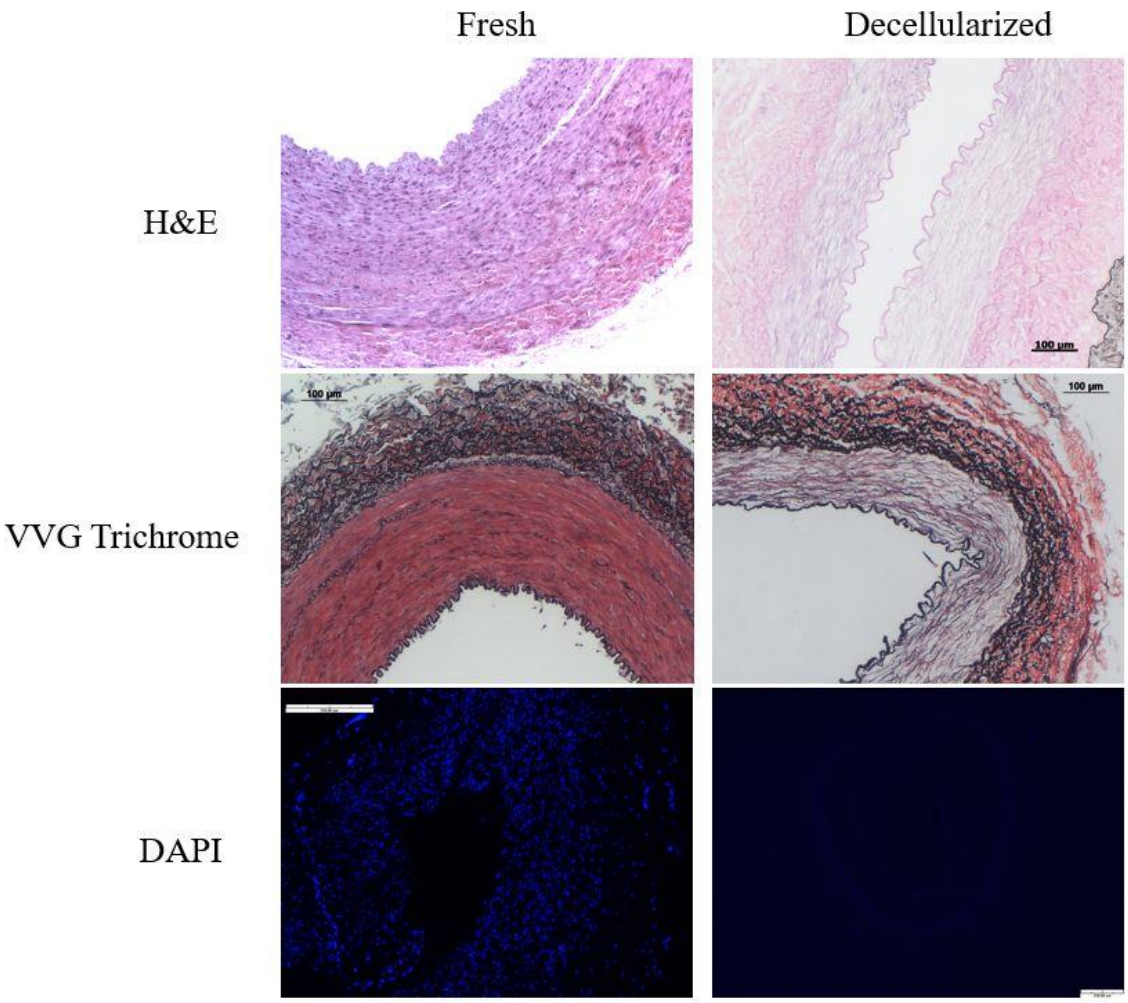


Fig. 3.1.2. Histological images of fresh and decellularized porcine renal arteries used in this study. For histological analysis, tissues were stained with Hematoxylin and Eosin (H&E, dark purple = nuclei, pink = background substance), VVG Trichrome (blue = collagen, black=elastin, red=muscle) and DAPI (fluorescent dots = nuclei).

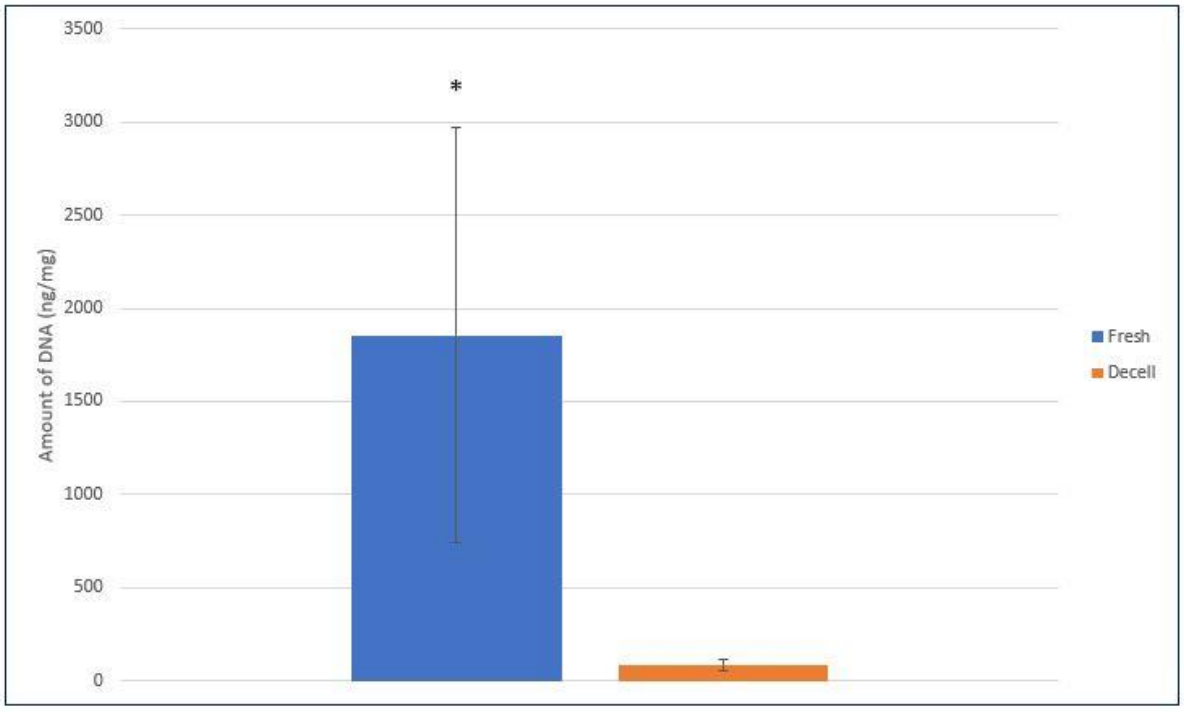


Fig. 3.1.3. Amount of DNA remaining in tissues after decellularization (Decell) process. A 95.6% reduction of DNA was achieved from Fresh to Decell. *Indicates statistical significance.

3.3.2. Mechanical Testing

Mechanical testing was crucial to ensure that the scaffolds had the mechanical properties to be physiologically appropriate for *in* vivo studies. In all of the mechanical tests performed, the results were not statistically different of PGG-treated and non-treated scaffolds compared to the fresh tissue controls. Uniaxial tensile testing (**Fig. 3.1.4)** showed that the renal artery scaffolds did not significantly alter their Young's Modulus value after decellularization. PGG-treatment slightly increased the Young's modulus, which was expected due to PGG binding to the collagen and elastin in the ECM. The fresh renal artery samples had an average Young's Modulus of 0.0015 mPa, the

decellularized renal artery samples had an average Young's Modulus of 0.0019 mPa, and the PGG-treated samples had an average Young's Modulus of 0.0025 mPa.

Suture retention strength testing (**Fig. 3.1.5)** using the uniaxial tensile test also showed a slight reduction of suture holding strength in the decellularized scaffold compared to the fresh tissue, but it was not a significant reduction to cause a noticeable difference in mechanical properties. The PGG-treated scaffold had the highest suture retention strength which indicates the increased stabilization and strength properties that the PGG treatment provides to the ECM.

Burst Pressure testing (**Fig. 3.1.6)** showed a slightly lower burst pressure in the decellularized and PGG-treated arteries compared to fresh arteries, however, the pressure values were above 2200 which were significantly higher than the expected physiological blood pressure values in the *in vivo* studies.

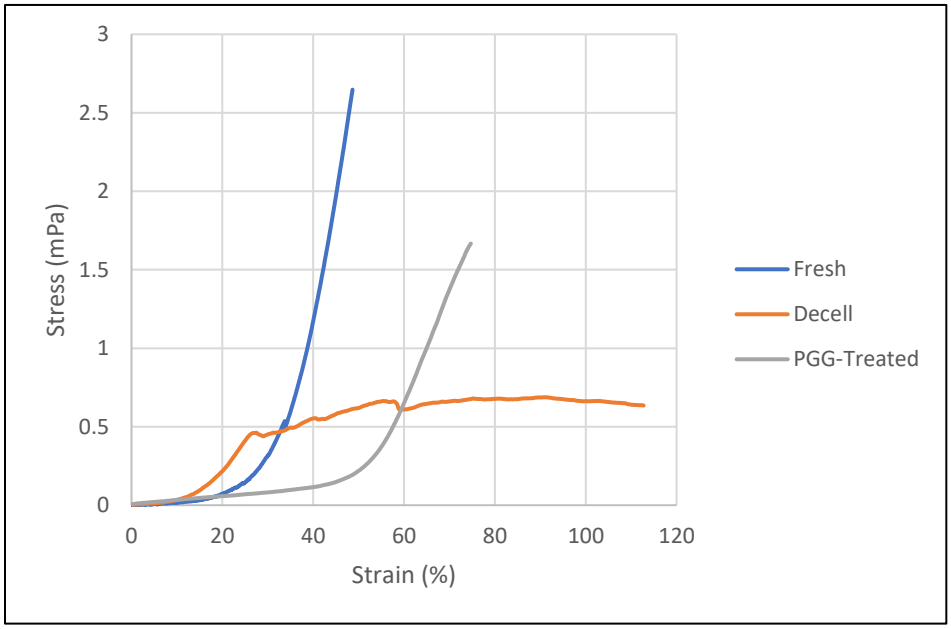
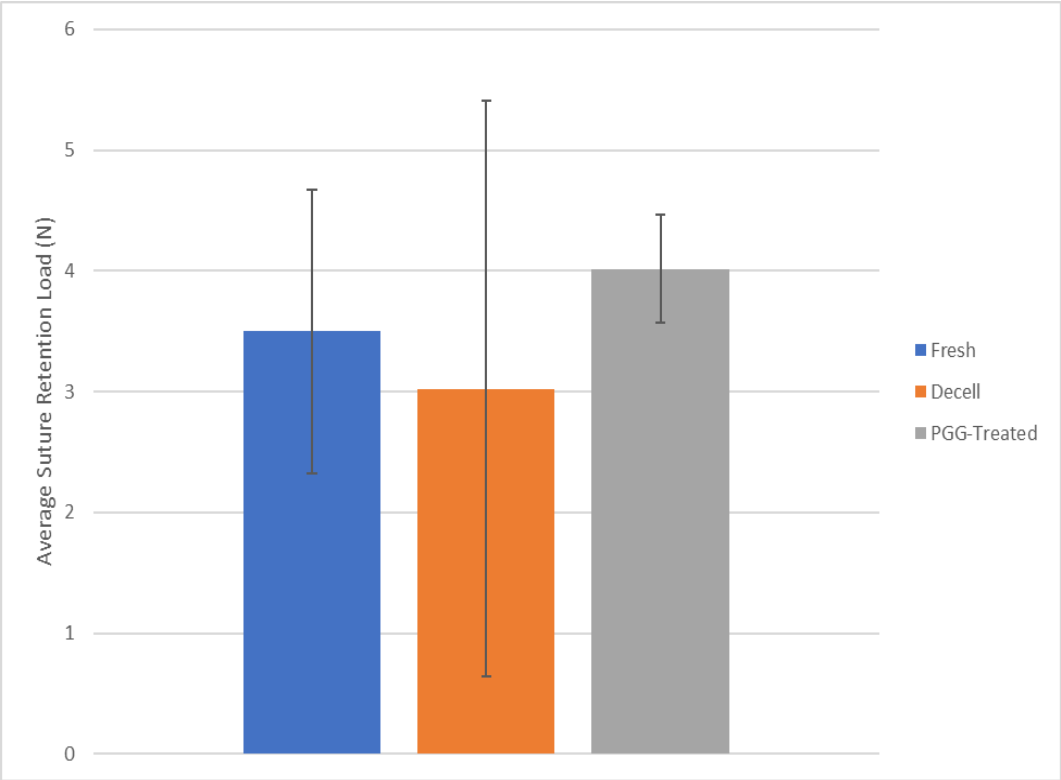
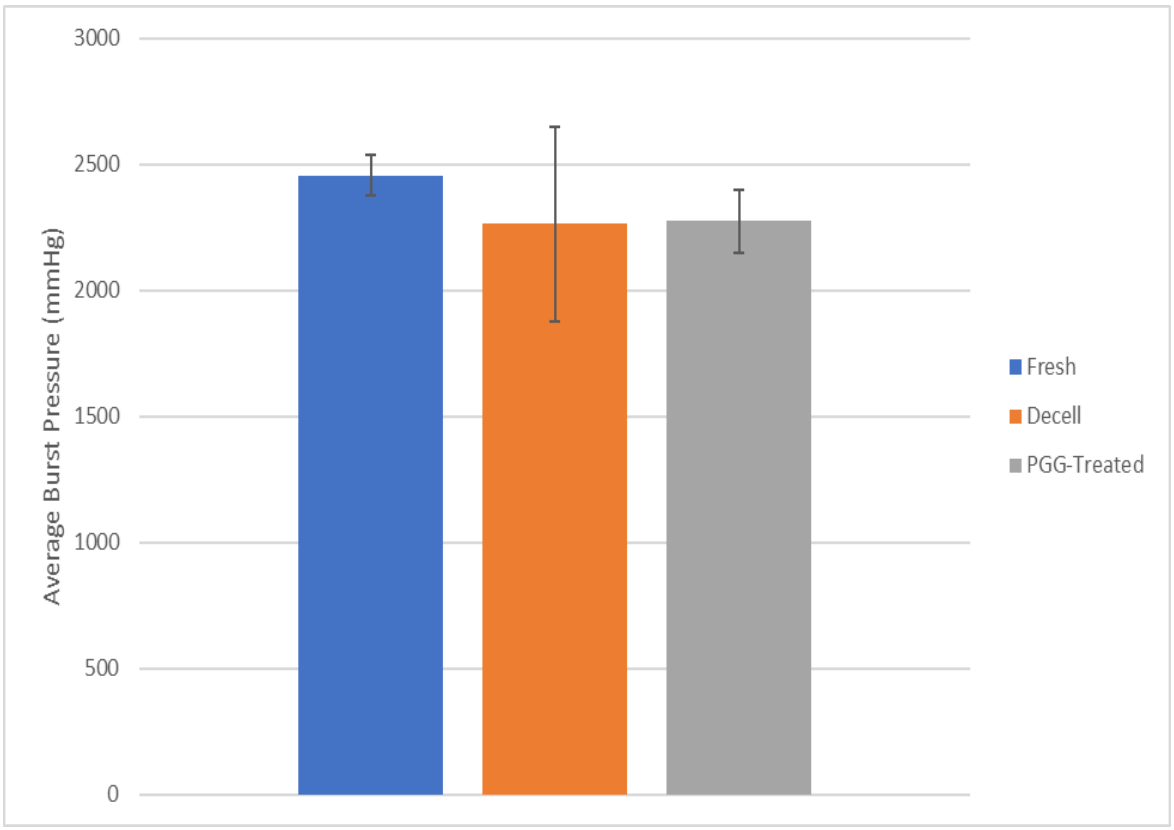


Fig. 3.1.4. Stress-Strain analysis using tensile testing with the MTS (n=4).



Scaffold Type	Average Suture Retention Load (N)	Standard Deviations
Fresh	3.5	1.17
Decellularized	3.0	2.38
PGG-Treated	4.0	0.4

Fig. 3.1.5. Average maximum suture retention load of fresh, decellularized and PGG-treated scaffolds. The results were not statistically different (n=4).



Scaffold Type	Average Burst Pressure (mmHg)	Standard Deviations
Fresh	2456	79.4
Decellularized	2263	385.5
PGG-Treated	2274	124.6

Fig. 3.1.6. Average burst pressure of fresh, decellularized and PGG-treated renal artery scaffolds. The burst pressure value was determined to be the water pressure at which the artery burst. The results were not statistically different (n=4).

3.4. Discussion

A completely ECM-based vascular scaffold devoid of any synthetic materials with preserved structure and mechanical integrity can be successfully engineered by decellularizing native blood vessels. Xenogeneic ECMs have already been used to replace or repair a variety of tissues and organs in both preclinical animal studies and human clinical applications. Specifically, the ECM derived from the porcine small intestinal submucosa (SIS) and urinary bladder submucosa (UBS) have been used as vascular graft materials. However, one of the major disadvantages to these ECM-based vascular grafts was the lack of sufficient mechanical performance to result in a long-term blood vessel replacement option. Similarly, the incorporation of natural and synthetic polymers have resulted in vascular scaffolds capable of the proper structural and functional features, however the limited proliferative ability of smooth muscle cells contributes to the poor *in vivo* mechanical performance.[2] Furthermore, the polymeric scaffolds may not interact with cells in the desired way since their surface chemistry does not promote adequate cell adhesion and it is generally difficult to construct a 3-D synthetic polymer scaffold that closely resembles the structure of natural tissues.[3] To construct scaffolds for this study, renal artery segments from porcine kidneys were chosen as the tissue source. The rationale behind this tissue source was to develop scaffolds with dimensions similar to rats' abdominal aorta, which was the intended location for implantation. Therefore, the arteries were segmented into 1-2 cm in length and 2-3 mm in diameter.

In addition to complete cell removal and a minimum 95% reduction of DNA, the resulting mechanical properties of the ECM including elastic modulus and tensile strength should match the original tissue.[4] These criteria were specifically focused on when developing the decellularization process for the development of our renal artery scaffolds. There are several different ways to decellularize tissues and the overall mechanism, advantages and disadvantages of each method were carefully considered when selecting the decellularizing agents for this study. The three main categories of agents commonly used are surfactants, acids and bases, and enzymes.[4,5] Mechanical and pressurized methods were not considered for this study.

Surfactants such as sodium dodecyl sulfate (SDS) have been successful in several decellularization applications, however, there have been instances in which SDS was damaging to the structural and signaling proteins; this was observed in SDS-treated heart valves, and human and porcine lungs.[4,6,7] GAGs and growth factors such as vascular endothelial growth factor (VEGF) can also be eliminated by SDS treatment, which can affect the biochemical cues regulating cell function. Damaged structural proteins can prevent proper cell infiltration as well as prevent the full preservation of its mechanical properties. Furthermore, while most surfactant-treated tissues can be washed with phosphate buffered saline, SDS is more difficult to remove due to its ionic nature. Thus, the extensive and time-consuming washing process following treatment is another disadvantage of SDS as a decellularizing agent. Triton X-100 is another commonly used surfactant that has shown high success in removing cells and DNA and is less harsh than

SDS, making it less damaging to the structural integrity of the tissue. It is also known to preserve collagen I as well as the tissues' mechanical properties.[4]

Peracetic acid is highly was used for sterilizing our scaffolds, however, it has also been used as a decellularizing agent by other research groups. Specifically, it has been applied to decellularization of SIS and the urinary bladder. However, complete cell removal was not achieved and mechanical properties were significantly altered in both applications.[4,8,9] Interestingly, the use of peracetic acid treatment on renal tissue has, in fact, demonstrated adequate preservation of ECM integrity and support for cell adhesion and healthy cell proliferation.[10] Due to the evidence that peracetic acid may not achieve complete cell removal, this agent was selected for sterilization purposes only since it was known that the structural properties would not be altered. Sterilization methods such as ethylene oxide exposure, gamma irradiation, and electron beam irradiation are known to alter ECM structure and mechanical properties.[11]

After consideration of all the different methods of decellularization, we selected 0.1M sodium hydroxide (NaOH) solution as the treatment agent for the renal arteries. One of the main reasons was the ease of making the solution and the short incubation period required to completely decellularize the tissue. Three hours of incubation under agitation was sufficient to remove the cells from the renal arteries, while other agents may require several hours or even days to achieve complete decellularization. Another main reason was that we wanted to focus on creating a primarily elastin-based scaffold, and NaOH is known to preserve the elastin content of tissues. Previous studies have explored the concept of developing a primarily collagen-based or primarily elastin-based

scaffold for improving cell migration after implantation. When approaches that preserved both collagen and elastin in aortic scaffolds was applied, cell migration into these decellularized tissues was inadequate, likely due to the very tight ECM organization of a complete collagen and elastin matrix.[3,12] By selectively removing matrix components from the decellularized tissue, the goal was to create a more porous scaffold which is capable of facilitating improved cell infiltration and repopulation.[3] Although many studies have focused on constructing collagen scaffolds, these scaffolds have displayed poor mechanical properties, including lack of elasticity. Furthermore, these studies show poor cell infiltration in decellularized aorta upon implantation, due to insufficient porosity and the dense structure of the arteries.[13] Based on this research, our approach was to selectively remove cells and collagen during the decellularization process with the goal of creating elastin-rich scaffolds. Elastin fibers provide vascular tissues with resilience, allowing long-range deformability. This is a critical property for the function of arteries, which undergo cyclical extension and recoil. Previously, it was shown that alkaline-extracted carotid arteries showed complete elimination of cells and almost complete collagen removal while maintaining elastin and proteoglycans intact.[13] For this reason, we decided to use NaOH instead of Triton-X 100 as the decellularizing agent since Triton-X 100 is known to specifically preserve collagen. Furthermore, it has been shown that for decellularization of renal tissues, NaOH has performed optimally in achieving complete cell removal, reduction of residual DNA to the immunogenic threshold suggested (50 ng/mg of dry tissue weight), preservation of elastin content, and high potential to encourage cell adhesion after seeding and host cell infiltration after

implantation.[10] Along with the NaOH treatment, enzymatic agents DNase and RNase were also used to ensure complete removal of DNA content from the ECM. These enzymes are typically used in combination with the main decellularizing agent because they cleave nucleic acid sequences and can therefore aid in removal of nucleotides after cell lysis in tissues.[4,11,14]

The results of this study confirmed that treatment with NaOH and DNase/RNase met all the criteria to achieve the goal of decellularization. Specifically, histological staining with H&E, VVG Trichrome, and DAPI provided qualitative data to show the absence of cells, and DNA quantification showed that the minimum threshold of residual DNA had been met (**Fig. 3.1.2, 3.1.3)**. This was an important criteria because presence of DNA is directly correlated to adverse host reactions.[11] VVG Trichrome staining was also used as an assessment method for observing alterations in the ECM; it was evident that the elastin fibers of the renal arteries were well preserved and even collagen appeared to be well maintained.

After verification of complete cellular removal, the effects of the decellularization procedure on the mechanical properties of the remaining ECM was the next area of interest. While there are a lot of conflicting results regarding the effects of different agents on various types of tissues, it is important to perform specific mechanical tests that are relevant to the intended application for the scaffolds.[11] For implantation in a rodent model, we performed tensile strength testing using stress-strain analysis, burst pressure testing and suture retention strength. As seen in **Fig. 3.1.4**, it is apparent that the elastic modulus of the tissues did not significantly change after decellularization. The PGG-

treated scaffolds showed a slight increase in tensile strength, indicating that the PGG-binding to the elastin stabilized and added strength to the scaffolds. The retention of sutures was another important mechanical criterion for successful implantation. If the implanted scaffold cannot hold a suture under physiological load and forces, the consequences of graft failure will be fatal to the host animal. **Fig. 3.1.5** shows the maximum suture retention strength of both scaffold types compared to the fresh, native renal artery. The non-treated decellularized scaffold only slightly decreased its suture retention strength compared to the fresh artery, indicating minimal alteration of mechanical structure and strength. The PGG-treated decellularized scaffold actually increased its suture retention strength, further proving that PGG-treatment strengthens the tissue while still preserving its native ECM architecture and mechanical integrity. Since these scaffolds were intended for implantation as a vascular graft, it was important to ensure that they could withstand the pressure of fluid flow and not rupture under physiological blood pressures. The maximum systolic blood pressures that could be observed in rats is 250 mmHg; **Fig. 3.1.6** shows that the scaffolds did not burst until greater than 2200 mmHg of water pressure, indicating that they were more than adequate for *in vivo* application as functional vascular grafts.

The comparison between PGG-treated scaffolds and non-treated scaffolds shows that although the decellularized tissues maintain adequate structural and mechanical properties compared to the native arteries, PGG-treated scaffolds are stabilized and may serve as better scaffold materials for *in vivo* applications because of their overall increase in tensile strength and suture retention strength. Specifically for the application in a

diabetic rat model, we chose to treat our decellularized scaffolds with PGG, a naturally derived polyphenol based on gallic acid, due to its antioxidant, anti-diabetic, and anti-inflammatory properties.[15–17] The exact mechanism by which PGG functions is not fully understood, but it may be based on the decrease of metal ions' uptake or the promoting of their excretion through chelating activities.[15] The motivation for incorporating PGG-treatment is based on the theory that scaffold pretreatments with antioxidants could improve the durability of vascular grafts in diabetes. PGG is known to have high affinity for proline-rich proteins, particularly collagen and elastin; and studies on rat aorta have shown increased stability of collagen and elastin and a slowed degradation by a factor of 50.[15,18,19] These characteristics of PGG provide a high potential for the stabilization of our ECM-based scaffolds and protection from diabetes related damage, thereby increasing their durability. The effects of PGG on the scaffolds in the diabetic environment will be further discussed in the following chapter.

3.5. Conclusion

Renal arteries obtained from porcine kidneys was an appropriate tissue source for developing vascular graft scaffolds for implantation at rats' abdominal aorta position. Decellularization methods using NaOH and DNase/RNase successfully removed all cellular material and DNA, while preserving elastin content and mechanical properties. This was confirmed with standard histological analysis and DNA quantification methods and mechanical testing for tensile strength, burst pressure, and suture retention strength. PGG-treatment of the scaffolds increased the overall durability of the scaffolds. The results from this study supported the use of the decellularized, PGG-treated scaffolds as

vascular graft implants in a diabetic rat model to study and determine their ability to resist diabetes-related alterations.

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CHAPTER 4

EVALUATING THE RESISTANCE OF PGG-TREATED VASCULAR GRAFTS TO DIABETES IN A CIRCULATORY SYSTEM OF AN ANIMAL MODEL

4.1 Introduction

The theory that a decellularized tissue scaffold can be transformed into a viable replacement for an inflamed, occluded, atherosclerotic or calcified cardiovascular tissue with just the addition of cells, growth factors and mechanical stimuli is a promising one but is far from reaching its true potential for clinical translation. This is due to the complicated implant-host interactions and chronic inflammation; and the presence of diabetes which further exacerbates these complications and reduces the success rate of the implanted tissue. The presence of diabetes and inflammation result in many complications such as stiffening, fibrosis and calcification.[1,2] Therefore, the main question that we must address is how to develop a TEVG that would be protected from diabetes-related damage.

Previously, subdermal implantation of ECM-based scaffolds treated with penta-galloyl glucose (PGG) in a diabetic rat model showed reduced AGE accumulation, calcification, infiltration of inflammatory cells, fibrosis, and stiffening in the scaffolds. At the same time, scaffold architecture and mechanical integrity was not disturbed.[3] We hypothesize that implanting PGG-treated scaffolds as vascular grafts in a diabetic rat model will similarly demonstrate such results. Demonstrating that this treatment method can resist diabetes-related damage in a functional, circulatory environment will have a significant impact on the research to develop a successful TEVG for diabetic patients. To test this hypothesis, PGG-treated TEVGs were implanted in Sprague-Dawley rats

induced with diabetes for 8 and 12-weeks. The grafts were monitored using ultrasound imaging to observe the blood flow and patency. After explanting the grafts, the tissues were characterized for modifications in ECM, inflammatory response, and overall remodeling using histology and protein analysis.

4.2. Materials and Methods

4.2.1. Vascular Graft Implantation

Adult male Sprague-Dawley rats (n=25, weight 300-350 g, Charles River) were ordered and allowed 10 days' time to acclimate. After the acclimation period, rats were prepped for surgery and anesthetized using 1-2% Isoflurane. After confirmation of completeness of anesthesia, invasive surgery through midline laparotomy incision was performed to expose the abdominal aorta using aseptic technique. A 20 mm piece of the aorta distal to the renal vein and artery and proximal to the iliac bifurcation was dissected and cleaned. 1 mL Heparin solution (50 Units/kg) was injected into the iliolumbar vein, and the graft was cut to required size. The aorta was clamped approximately 15 mm apart, and a 10 mm section of aorta was removed. The exposed aortic lumens were washed with Heparin solution and 12-15 interrupted sutures (distal first) using 8-0 Nylon. Before starting proximal suturing, the graft was filled with saline solution. Suturing was repeated for the proximal anastomosis. The distal clamp was released first followed by the proximal clamp. After bleeding has been contained (no more hemorrhage) the graft was assessed for an additional 5-10 minutes, observing for adequate pulsation and patency. The color of the lower limbs was also observed for patency assessment. The

abdomen was closed in layers and buprenorphine (0.1 mg/kg) was administered subcutaneously twice daily for 3 days. The incision was closed with surgical staples and the rats were recovered and provided with food ad libitum. The entire procedure required 75-90 minutes for each rat.

After 8 weeks, the rats were humanely euthanized by CO_2 asphyxiation and the vascular grafts were explanted and collected according to their respective application as follows: grafts for histological analysis were placed in 10% phosphate-buffered formalin and paraffin embedded; and samples for protein analysis were flash frozen in liquid nitrogen and kept on dry ice until transferred to -80℃ for storage.

A second study was conducted for 8 and 12-weeks of implantation using the same surgical procedure described above, with 8 diabetic rats and 8 non-diabetic rats. The same methods and parameters were used to process the explanted grafts as the first 8-week study.

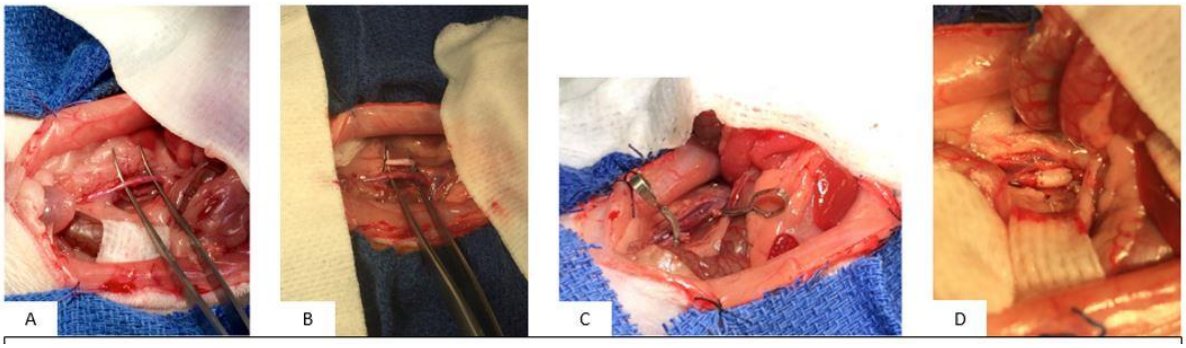

Fig. 4.1.1 Steps of the vascular graft implantation procedure as an end-to-end anastomosis in the abdominal aorta of a Sprague-Dawley rat. (A) The abdominal aorta is isolated, (B) the size and length of the graft is assessed to ensure compatibility with the native vessel, (C) the abdominal aorta is clamped at the proximal and distal ends to stop blood flow and cut, (D) the graft is sutured to both ends of the aorta and pulse is observed to confirm proper blood flow through the graft.

4.2.2. Rat Model of STZ-induced Diabetes

Two weeks after vascular graft implantation, the rats (n=9) were induced with diabetes by administering a single dose of sterile filtered 55 mm/kg streptozotocin (STZ) solution in 0.1M citrate buffer (pH 5) by tail vein injection. Control rats (n=13) received an equal volume of sterile citrate buffer. After 72 hours from STZ administration, blood glucose levels were measured 3-4 times per week, using AlphaTRAK (Gen II) test strips on the AlphaTRAK Blood Glucose Monitoring System which is specifically designed for animals. Diabetic rats (>250 mg glucose/dL blood) received subcutaneous injections of long-lasting insulin (2-4 Units Isophane) every other day to maintain blood glucose level in a desirable range (250-600 mg glucose/dL blood) and prevent development of ketonuria and weight loss. Glucose levels, individual weights, hydration status, and food and water consumption were monitored closely and continuously graphed to ensure adequate health parameters. Animals were provided with food and water ad libitum and were cared for by the attending university veterinarian and associated staff at the Godley-Snell Research Center animal facility. The animal protocol was approved by the Animal Research Committee at Clemson University, and National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication #86-23 Rev. 1996) were observed throughout the experiment.

Insulin Dosage	
Glucose level (mg/dl)	Units of Insulin Administered
200-300	2
300-500	3
500-700+	4

Table. 4.1. The general guidelines followed for insulin dosage based on glucose measurements

Study 1

	Rat #	Graft Type
CONTROL	7	Non-PGG
	8	Non-PGG
	10	Non-PGG
	19	Non-PGG
DIABETIC	2	PGG
	15	PGG
	17	PGG
	18	PGG
	20	Non-PGG
	21	Non-PGG
	22	Non-PGG
	24	Non-PGG
	25	Non-PGG

Study 2

	Rat #	Graft Type	Time Point
DIABETIC	3	Non-PGG	2 mon
	5	Non-PGG	3 mon
	6	Non-PGG	3 mon
CONTROL	9	Non-PGG	2 mon
	10	Non-PGG	3 mon
	11	Non-PGG	3 mon
	13	PGG	2 mon
	14	PGG	2 mon
	16	PGG	3 mon
	17	PGG	3 mon
DIABETIC	19	PGG	2 mon
	22	PGG	2 mon
	23	PGG	3 mon
	24	PGG	3 mon

Tables 4.2. (left) and 4.3. (right). Experimental implant groups for the two rat studies conducted; (**4.2)** 8-week study with 4 non-treated grafts, non-diabetic control, 4 treated grafts, diabetic, and 5 non-treated grafts, diabetic. (**4.3)** 8 and 12-week study; 8-week groups: 1 diabetic non-treated graft, 1 non-diabetic PGG-treated graft, 2 non-diabetic PGG grafts and 2 diabetic PGG grafts. 12-week groups: 2 diabetic non-treated grafts, 2 non-diabetic non-PGG grafts, 2 non-diabetic PGG grafts, and 2 diabetic PGG grafts.

4.2.3. Ultrasound Imaging

To observe the patency, blood flow, and overall condition of the vascular grafts after implantation, Ultrasound imaging was performed to visualize the grafts using the Vevo 2100 system from VisualSonics. The rats' abdominal aorta was imaged prior to surgery to establish a normal, native view of the arterial blood flow and pulsatility. The rats were allowed one week to recover after the surgery before beginning imaging. The rats were placed under light anesthesia (2-3% Isoflurane) and prepped by shaving and cleaning the abdominal region. Then, the rat was laid supine on the system platform and its limbs secured with tape and electrode gel (for heart rate monitoring) in the anatomical position for rodents. The MS250 transducer was used to image the abdominal region and identify the abdominal aorta and implanted graft. Images were recorded in the color doppler mode, pulse wave mode (PW mode) and B-mode. The color doppler images allowed visualization of arterial blood flow from the abdominal aorta through the graft (red) as well as venous blood flow (blue); the PW mode showed the pulsation of the graft and allowed recording of the QRS complex to determine strength of blood flow; B-mode was used to obtain still images. The grafts were imaged at three different time points: 2-weeks post-surgery, mid-way through the implantation period, and just before explanting the graft.

Fig. 4.1.4. Ultrasound set-up

4.2.4. Histological Analysis

Paraffin sections (5 µm) were rehydrated and stained with Hematoxylin and Eosin (H&E) for a general analysis of ECM morphology and host cell infiltration during implantation. A combination Verhoeff van Gieson Trichrome stain (VVG Trichrome), derived from the individual VVG and Masson's Trichrome staining protocols, was used to further assess ECM composition and collagen and elastin integrity.

Immunohistochemistry (IHC) was performed for detection of various cellular markers in the explanted grafts. The results were compared to the native abdominal aorta as well as analyzed for differences between diabetic and non-diabetic and PGG-treated and non-treated grafts. Briefly, rehydrated paraffin sections (5 µm) were exposed to a boiling water bath in a pressure cooker for 5 minutes with citrate buffer, pH 6, or tris-based buffer, pH 9 (Vector Laboratories, Birmingame, CA), depending on the method specified by the antibody product datasheet, to unmask the antigens. If enzyme-mediated antigen retrieval was specified, the sections were exposed to 0.1% Proteinase K in 10

mM TRIS buffer, pH 7.5, at room temperature for 30 seconds. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 0.3% normal horse serum. Sections were treated with 0.025% Triton X-100 for 10 minutes and then incubated with normal blocking serum for 20 minutes. Primary antibodies were applied and kept overnight at 4°C. Negative staining controls were obtained by the omission of the primary antibody. The Vector ABC peroxidase substrate kit followed by the DAB kit was then used to visualize the antibody staining, and sections were lightly counterstained with Hematoxylin, prior to mounting. Digital images were obtained at various magnifications (25 to 100) on a Zeiss Axiovert 40CFL microscope using AxioVision Release 4.6.3 digital imaging software (Carl Zeiss MicroImaging, Inc. Thornwood, NY).

4.2.5. Evaluation of Vascular Cell Infiltration

To determine if vascular cells were infiltrated in the scaffolds by the host, IHC was performed following the protocol described above, and using specific antibodies for endothelial cells (vWF, Abcam 6994, 1:1000 dilution), smooth muscle cells, (αSMA, Abcam 7817, 1:500 dilution) and fibroblasts (HSP47, Abcam 77609, 1:200 dilution).

4.2.6. Evaluation of Infiltrated Cells

To identify the cells infiltrated in the scaffolds by the host, IHC was performed following the protocol described above and using specific antibodies for T-lymphocytes (CD8, GeneTex GTX76644, 1:100 dilution), macrophages (CD68, Abcam 31630, 1:100 dilution), M2 macrophages (CD163, Abcam 182422, 1:500 dilution) and M1 macrophages (iNOS, Abcam 15323, 1:100 dilution).

4.2.7. AGE and Oxidative Stress Detection

To determine the presence of AGE formation and oxidative stress in the grafts, IHC was performed following the protocol described and using antibodies for AGE (Abcam 23722, 1:1000 dilution) and NADPH-oxidase 4, an oxidative stress marker (Abcam 133303, 1:500 dilution).

4.3 Results

4.3.1. Induction and Monitoring of Diabetes in Rats

Administration of STZ successfully induced diabetes in the rats after 72 hours. Glucose measurements were taken every other day and consistently indicated glucose levels in the range of 450-600 mg/dL in the rats injected with STZ, and glucose levels of 100 mg/dL-150 mg/dL in the control rats. Weight was also measured along with glucose as an assessment of overall rat health. The diabetic rats' weights ranged from 430-490 grams which was lower than the Control rats which weighed between 495-560 grams at a lower range than the Control rats. A weight loss greater than 20% of the day zero weight (day of surgery) was considered an indication of poor rat health and euthanasia was advised. Most of the STZ-treated rats lost approximately 10-15% of their weight but overall maintained healthy weights with proper insulin administration.

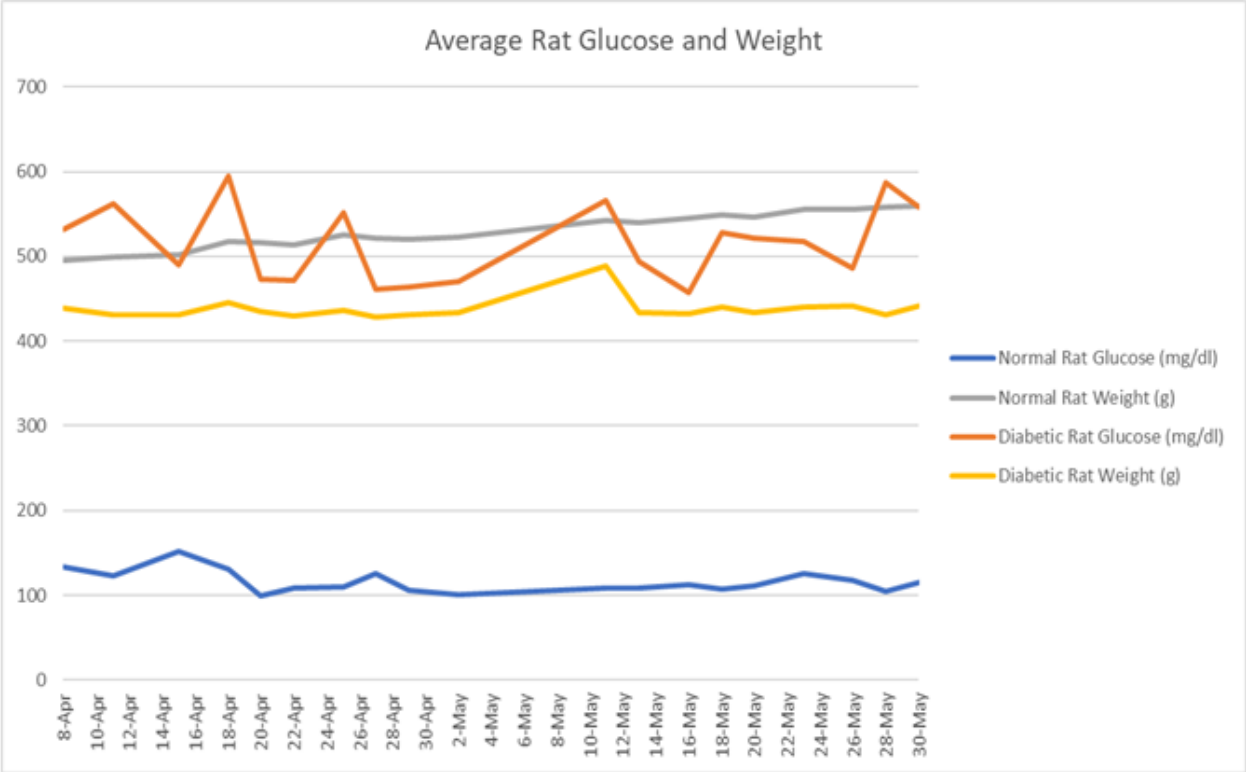

Fig. 4.1.5. Graph of average glucose and weight measurements of diabetic and normal (control) rats. The measurements were taken over a 12-week implantation period beginning at 72 hours post STZ administration.

4.3.2. Analysis of Graft Patency Based on Ultrasound Images

The Vevo 2100 Ultrasound system was a valuable tool that we used to observe the status of the implanted vascular grafts and assess the overall patency and integration of the grafts with the native vasculature of the rats. Interestingly, most of the PGG-treated grafts remained patent while the majority of non-PGG grafts did not maintain patency. This was determined based on 3 observations: the ability to visualize the graft on the ultrasound, the flow of blood indicated in red by the color doppler, and the of strength pulse wave. **Fig. 4.5** shows examples of what was determined to be a patent graft and

non-patent graph. **Tables 4.3 and 4.4** indicate the rats whose grafts remained patent and non-patent by the 2 and 3-months' time point.

2 Months	Rat #	Graft Type	Patent	Non-Patent
CONTROL	7	Non-PGG		X
	8	Non-PGG		X
	9	Non-PGG	X	
	10	Non-PGG	X	
	19	Non-PGG		X
	13	PGG	X	
	14	PGG		X
DIABETIC	2	PGG	X	
	15	PGG	X	
	17	PGG	X	
	18	PGG		X
	19	PGG	X	
	22	PGG		X
	3	Non-PGG		X
	20	Non-PGG		X
	21	Non-PGG		X
	22	Non-PGG		X
	24	Non-PGG	X	
	25	Non-PGG	X	

Group (2-months)	% of Patent Grafts
Control, Non-PGG	40
Control, PGG	50
Diabetic, Non-PGG	33
Diabetic, PGG	67

3 Months	Rat #	Graft Type	Patent	Non-Patent
Diabetic	5	nonPGG	X	
	6	nonPGG		X
Control	10	nonPGG	X	
	11	nonPGG	X	
	16	PGG	X	
	17	PGG	X	
Diabetic	23	PGG	X	
	24	PGG		X

Group (3-months)	% of Patent Grafts
Control, Non-PGG	100
Control, PGG	100
Diabetic, Non-PGG	50
Diabetic, PGG	50

Tables 4.4 and 4.5. Patent and non-patent grafts in each group as observed on the Ultrasound. (**Left**) Green=Non-PGG grafts, Blue=PGG grafts.

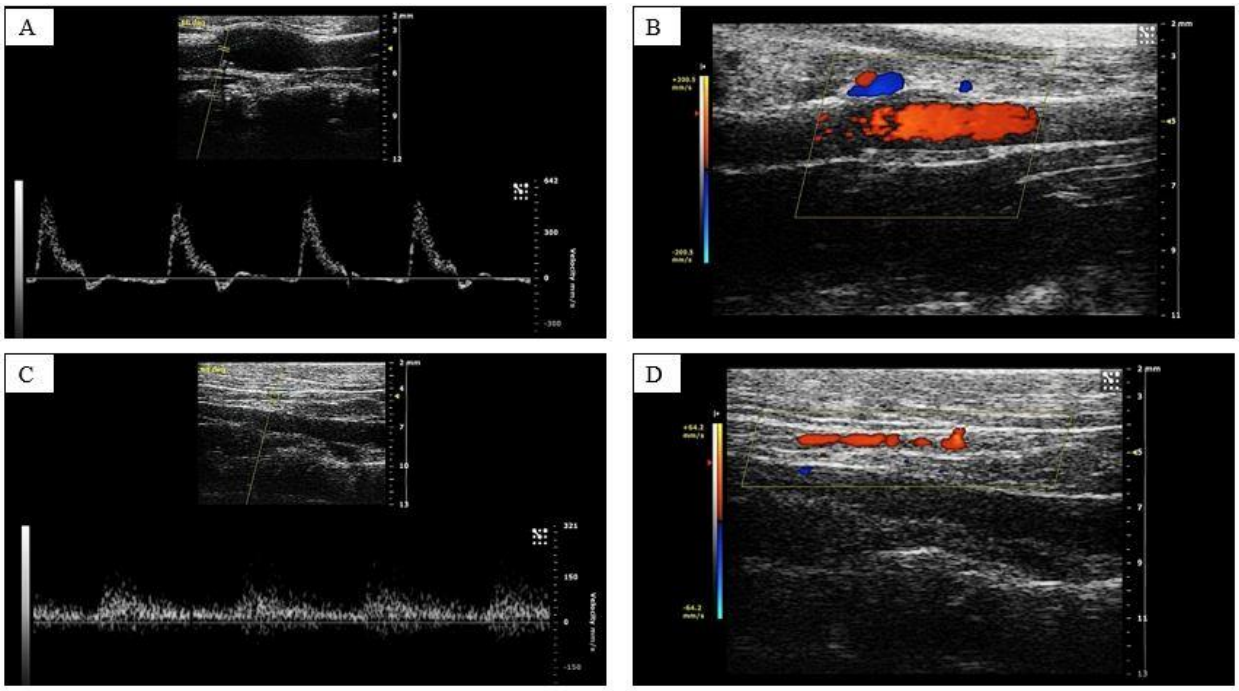

Fig. 4.1.6. Examples of patent and non-patent vascular grafts at the abdominal aorta site of the rats. (A) Patent graft indicated by a clear pulse wave and the strong red signal on the color doppler; (C, D) non-patent graft indicated by visually unclear outline of the graft and unclear pulse wave and a weak color doppler signal.

4.3.3. Explant and Evaluation of the Vascular Grafts

At the end of each time-point (8 and 12-weeks after induction of diabetes) the rats were euthanized, and the abdominal cavity was exposed to find the implanted graft. In general, the tissue surrounding the implant site look healthy, based on the pink and reddish tones of the tissue. The implanted grafts remained at their position and the sutures were intact. No signs of blood clotting or hemorrhaging was observed. It was interesting to see that while most of the grafts maintained their structural integrity, some of them did not and in some cases, had completely degraded. In this case, the tissue did

look unhealthy, indicated by pale, brown or dark coloring of the surrounding tissue. The degraded grafts directly correlated with the same grafts that appeared non-patent on the Ultrasound images. The number of PGG treated grafts and non-treated grafts that remained structurally intact and patent was assessed to see if there were differences and also compared to diabetic and non-diabetic conditions. The initial qualitative analysis indicated that the PGG-treated grafts maintained their structure and patency much better than the non-treated grafts, further confirming the patency assessment from the ultrasound images.

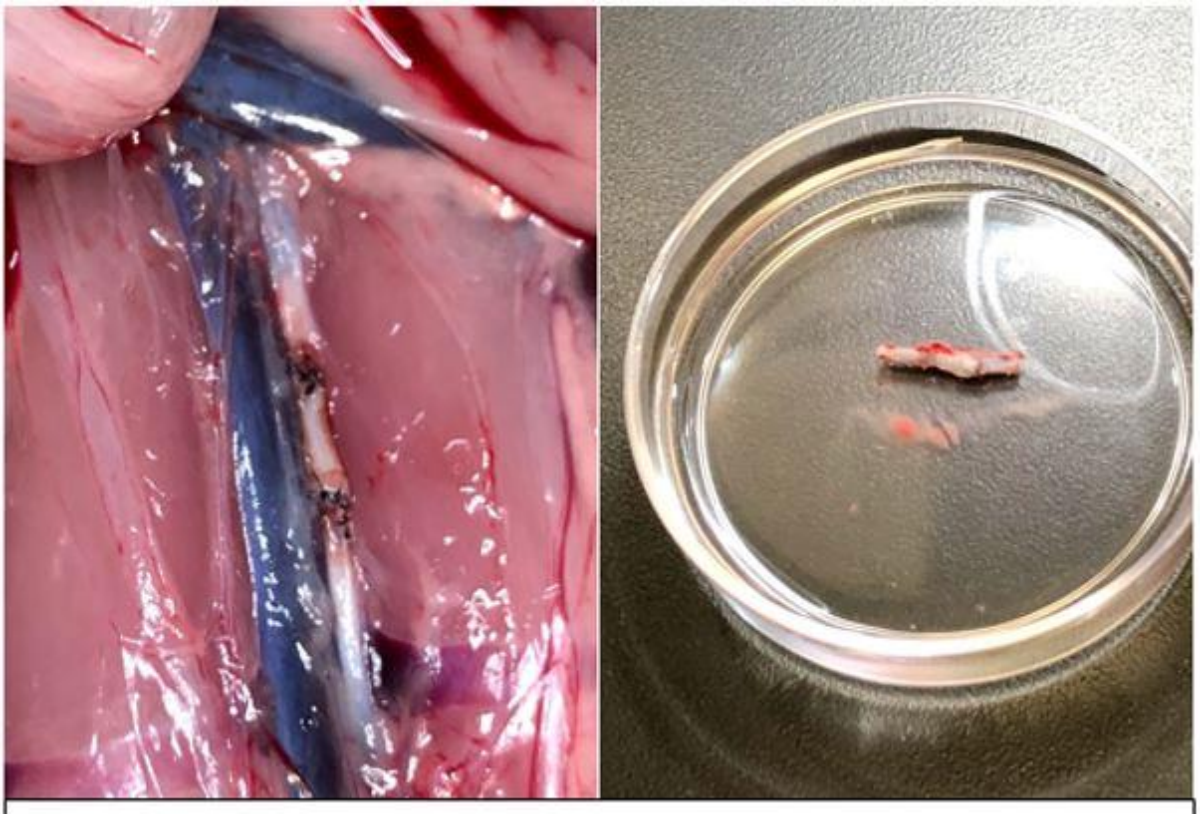

Fig. 4.1.7. Macroscopic images of the implanted vascular graft before (left) and after (right) explanting.

4.3.4. Histological Analysis of Host Cell Infiltration and Extracellular Matrix Remodeling

To determine how the diabetic host reacted to the implanted grafts, H&E and a combination VVG Trichrome staining was performed to observe host cell infiltration and modifications in the ECM. The explanted vascular grafts from each group showed a positive remodeling response and integration with the native vasculature in both 2 and 3-month time points. H&E staining showed significant host cell infiltration in the adventitia, media and intima of the grafts (**Fig. 4.1.8)**. The adventitia appeared to have the most cell infiltration compared to the media. Qualitatively, no differences were observed in cell infiltration patterns between PGG-treated and non-treated grafts or diabetic and control, non-diabetic rats. The combination VVG Trichrome staining showed excellent preservation of elastin fibers in the grafts from all groups at the 2-month time point. Some degradation of elastin was observed in the grafts from the 3-month time point, especially in the non-treated grafts, but the elastin in the PGG-treated grafts remained adequately preserved. The presence of diabetes did not result in significant elastin degradation at the 2-month time point, however, elastin degradation was apparent at the 3-month time point in the diabetic non-treated grafts. PGG treatment seemed to be very helpful at preserving elastin under diabetic conditions even at the 3-month time point. Collagen fibers, although lightly visible, were not a dominant component of the scaffold ECM based on the VVG Trichrome staining. It was determined that the implanted scaffolds, both PGG-treated and non-treated, encouraged cell infiltration and the PGG-treated scaffolds showed overall better preservation of elastin compared to the non-treated scaffolds especially under diabetic conditions at both

2 and 3-month time points. Furthermore, scaffold structural integrity and ECM components remained well maintained between the 2 to 3-month time points; the only alteration observed was the slight degradation of elastin at the 3-month time point.

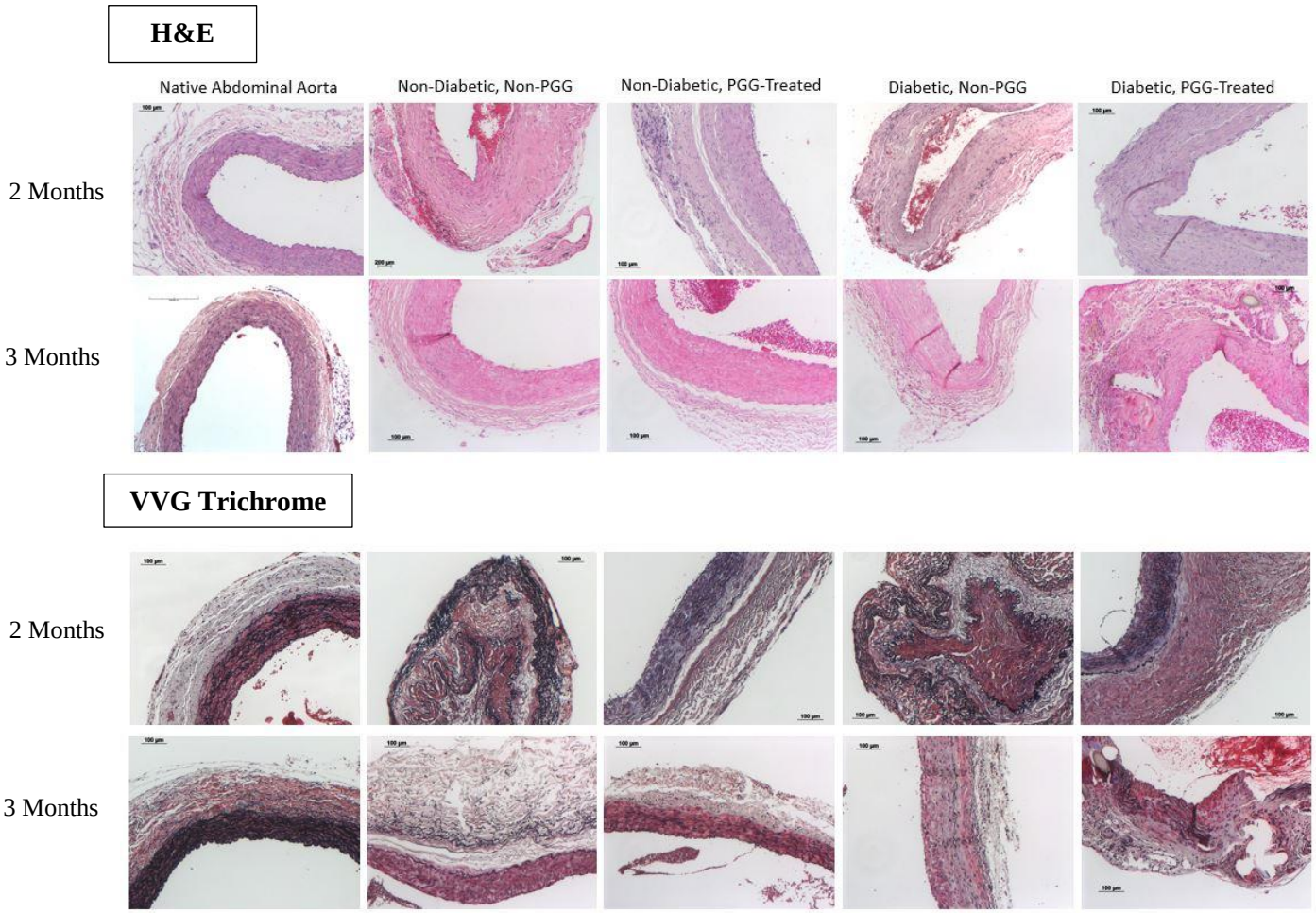

Fig. 4.1.8. Histological images of explanted grafts with and without PGG-treatment in diabetic and non-diabetic conditions. Tissues were stained with Hematoxylin & Eosin (**Top:** dark purple=cell nuclei, pink=extracellular matrix), and combined Verhoeff van Gieson and Masson's Trichrome (**Bottom:** black= elastin fibers, blue= collagen, red= muscle).

4.3.5. Immunohistochemical Analysis of Vascular Cell Infiltration

Following analysis of the vascular graft ECM and general host cell infiltration patterns, we investigated whether the grafts were able to repopulate with vascular cells. IHC was performed to identify presence of endothelial cells and smooth muscle cells and whether a complete endothelial layer in the intima and smooth muscle layer in the media was developed. IHC staining for von Willebrand factor (vWF), an endothelial cell marker, revealed that all of the grafts developed a continuous endothelial layer in the lumen. Staining for α-smooth muscle actin (α-SMA), a smooth muscle cell marker, showed formation of a smooth muscle layer in the media of the grafts. There was no noticeable difference in the vascular cell infiltration patterns between PGG-treated and non-treated grafts and PGG-treatment did not inhibit vascular cell infiltration.

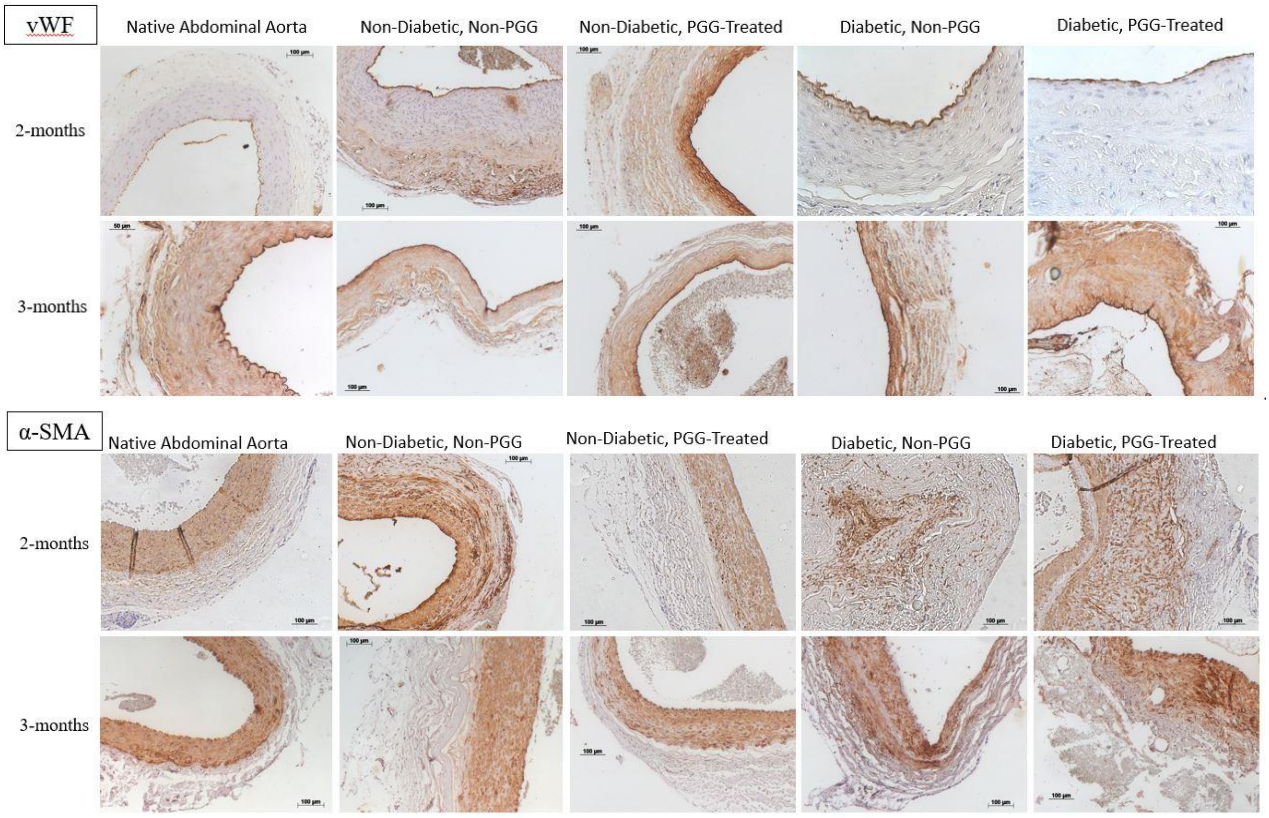

Fig. 4.1.9. Endothelial and smooth muscle cell infiltration in vascular grafts. The explanted tissues were stained for von Willebrand factor (vWF, endothelial cells) and α-smooth muscle actin (α-SMA, smooth muscle cells). Taken at 100X magnification, Brown=positive expression of marker, blue=cell nuclei.

4.3.6. Immunohistochemical Analysis of Inflammatory Cell Infiltration

Gaining an understanding of the host's inflammatory response to the implanted grafts was a very important aspect of this study. Specifically, we wanted to observe differences in diabetic and control, non-diabetic conditions and learn whether PGG-treatment affects inflammatory cell infiltration. T-lymphocytes and macrophages are major players in the inflammatory response and the specific markers for these cells were detected using IHC (**Fig. 4.2.1)**. Staining for CD8, a T-lymphocyte (T-cells) marker,

showed presence of very few T-cells in all scaffolds, but they were slightly more expressed in the non-treated grafts compared to the PGG-treated grafts. Diabetic conditions appeared to elicit a slightly higher T-cell response in the non-treated grafts at both the 2 and 3-month time point. Overall, the PGG-treated grafts appeared to discourage T-cell infiltration in both diabetic and non-diabetic environments. Furthermore, CD8 expression seemed to decline from 2 months to 3 months, marking a decrease in the presence of T-cells in the tissues (**Fig. 4.2.2)**. To study macrophage infiltration patterns, we stained for CD68, a pan-macrophage marker. Macrophages were highly expressed, in all of the graft samples, however, the diabetic PGG-treated grafts had greater infiltration of macrophages compared to the non-diabetic, non-treated grafts at both the 2 and 3-month time points. There was not a significant difference in macrophage expression between the PGG-treated and non-treated grafts in the non-diabetic rats, and interestingly, the diabetic non-treated grafts had the lowest expression of macrophages at the 2-month time point. However, by the 3-month time point, the diabetic non-treated graft showed an increased expression of CD68 compared to the non-diabetic, non-treated grafts, which showed the lowest CD68 expression at the 3-mont time point. To further investigate whether the macrophages were of the M1 (pro-inflammatory) or M2 (pro-remodeling) phenotype, the samples were stained for iNOS and CD163, markers for M1 and M2 macrophages, respectively (**4.2.3**. At the 2-month time point, it was apparent that the non-treated grafts in the diabetic rats had a highest expression of iNOS, while the diabetic PGG-treated grafts showed the highest expression of CD163. Overall, the PGG-treated grafts showed higher expression of CD163 in both

diabetic and non-diabetic conditions. iNOS expression was generally lower in the non-diabetic conditions and there was not a significant difference between the PGG-treated and non-treated grafts. iNOS expression seemed to be higher in the diabetic rats in both PGG-treated and non-treated grafts at the 2-month time-point.

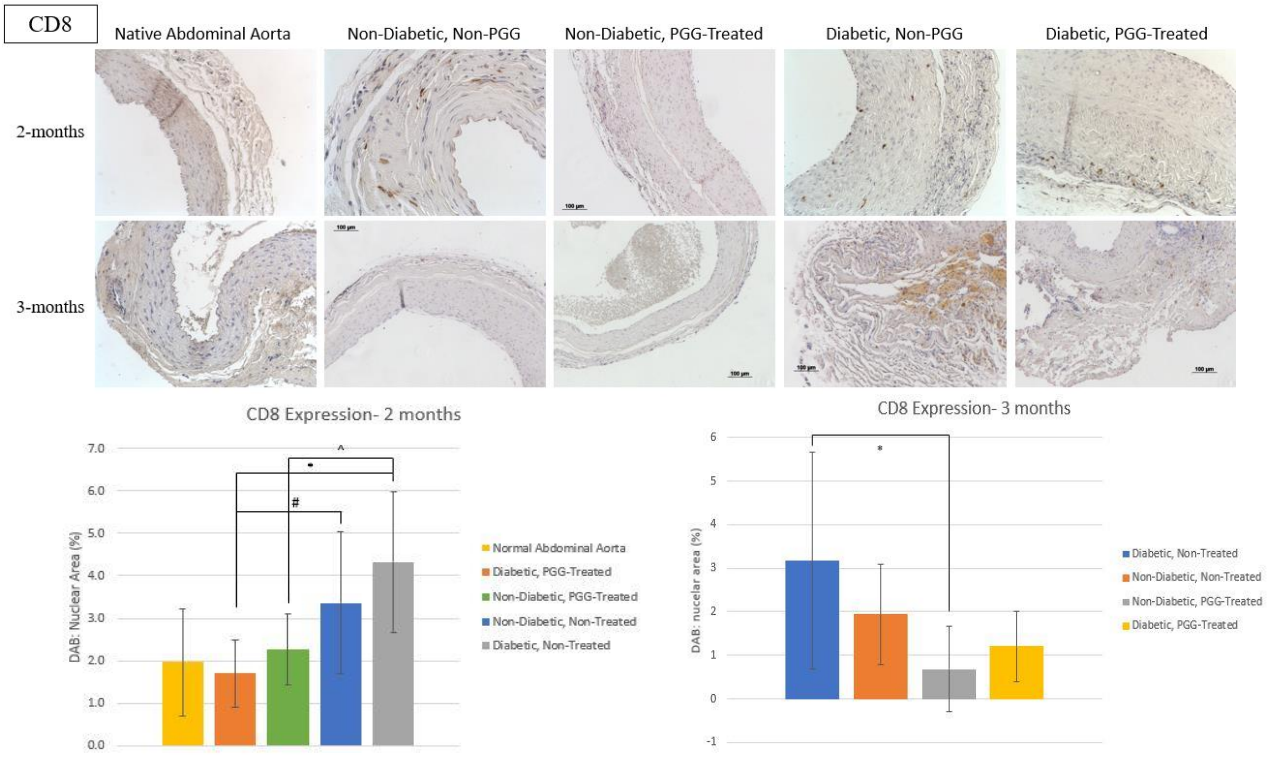

Fig. 4.2.1 Immunohistochemistry and Semi-Quantitative Analysis of CD8 (T-cells) Expression. Expression of CD8 at both the 2 and 3- month time points is shown as well as treatment and tissue type (n=6); images taken at 100X magnification (Brown=positive expression of markers, Blue=cell nuclei). Semi-quantification analysis (n=6) shows differences in CD8 expression between the groups. (^, *, # indicates statistical difference between the groups indicated)

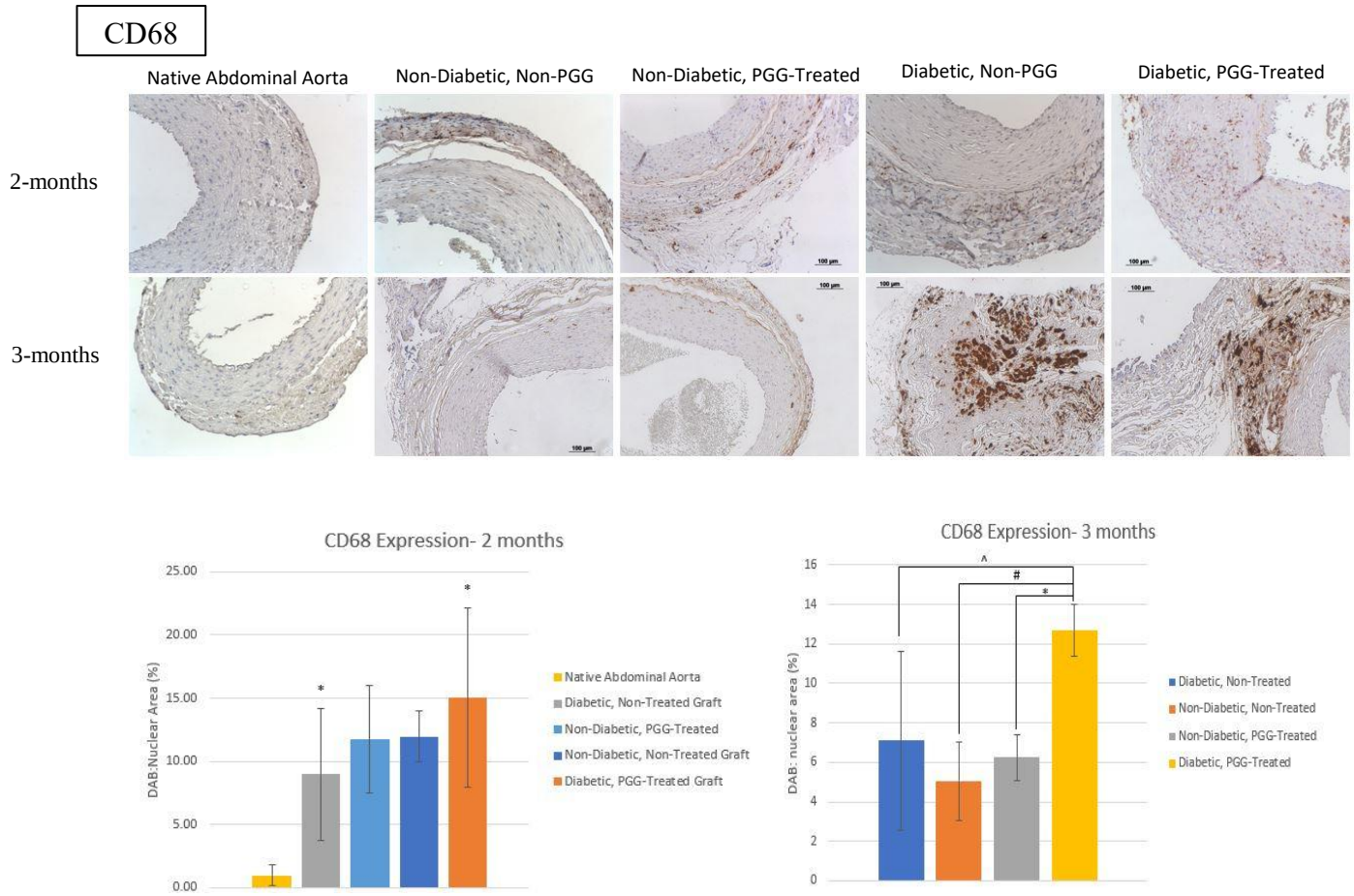

Fig. 4.2.2. Immunohistochemistry and semi-quantitative analysis of CD68 (pan-macrophages) expression. Expression of CD68 at both the 2 and 3- month time points is shown as well as treatment and tissue type; images were taken at 100X magnification (Brown=positive expression of markers, Blue=cell nuclei). Semi-quantification analysis (n=6) shows differences in CD68 expression between the groups. (^, #, * indicates statistical difference between the groups indicated).

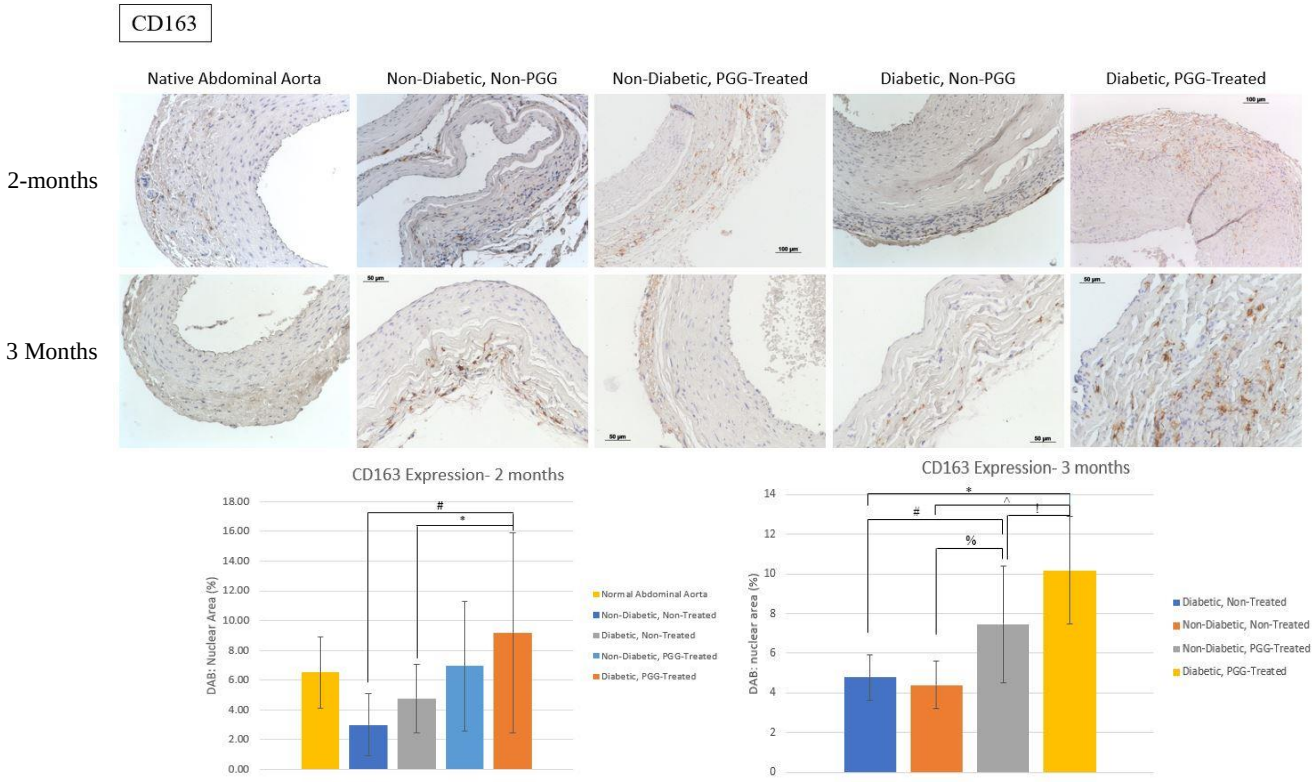

Fig. 4.2.3. Immunohistochemistry and semi-quantitative analysis of CD163 (M2 macrophages) expression. Expression of CD163 at both 2 and 3-month time points is shown as well as treatment and tissue type. (Brown=positive expression of CD163). Images were taken at 100X (top panel) and 200X (bottom panel).

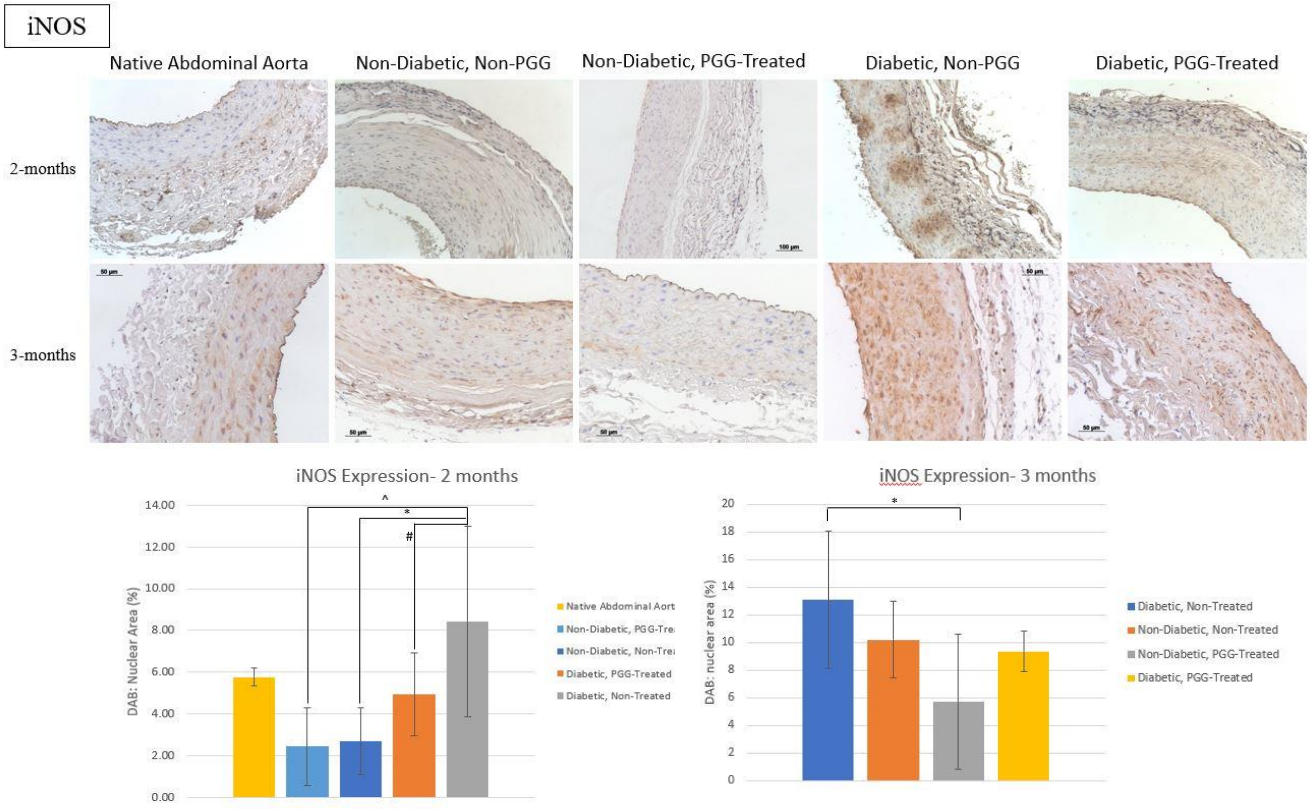

Fig. 4.2.4. Immunohistochemistry and semi-quantitative analysis of iNOS (M1 macrophages) expression. Expression of iNOS at both 2 and 3-month time points is shown as well as treatment and tissue type. (Brown=positive expression of iNOS). Images were taken at 200X (^, *, # indicates statistical differences).

4.3.7. Immunohistochemical Analysis of AGEs and Oxidative Stress

Following analysis of inflammatory cell infiltration in the explanted vascular grafts, we wanted to observe AGE accumulation and oxidative stress in the tissues and whether the PGG-treated scaffolds inhibited formation of AGEs and oxidative stress. To test this, IHC staining with AGE and NADPH-oxidase 4 antibodies was performed. As seen in **Fig. 4.2.5.**, the PGG-treated scaffolds showed very sparse, if any, expression of NADPH-oxidase 4 at both the 2 and 3-month time points. There was significant

expression of this marker in the non-treated grafts, especially in the diabetic environment, although the grafts from the 3-month time point showed decreased expression of NADPH-oxidase 4 compared to the 2-month time point. The expression of NADPH-oxidase 4 was also negligible in the non-diabetic grafts and there was no significant difference between the PGG-treated and non-treated grafts.

AGE accumulation was observed to some degree in all of the grafts, but the diabetic non-treated grafts showed the highest accumulation of AGEs. In general, the non-treated grafts exhibited higher AGE accumulation compared to the PGG-treated scaffolds, which appeared to inhibit accumulation of AGEs. Although AGE accumulation slightly increased between the 2-month time point to the 3-month time point, the PGG-treated scaffolds continued to inhibit AGE accumulation more than the non-treated scaffolds under diabetic conditions.

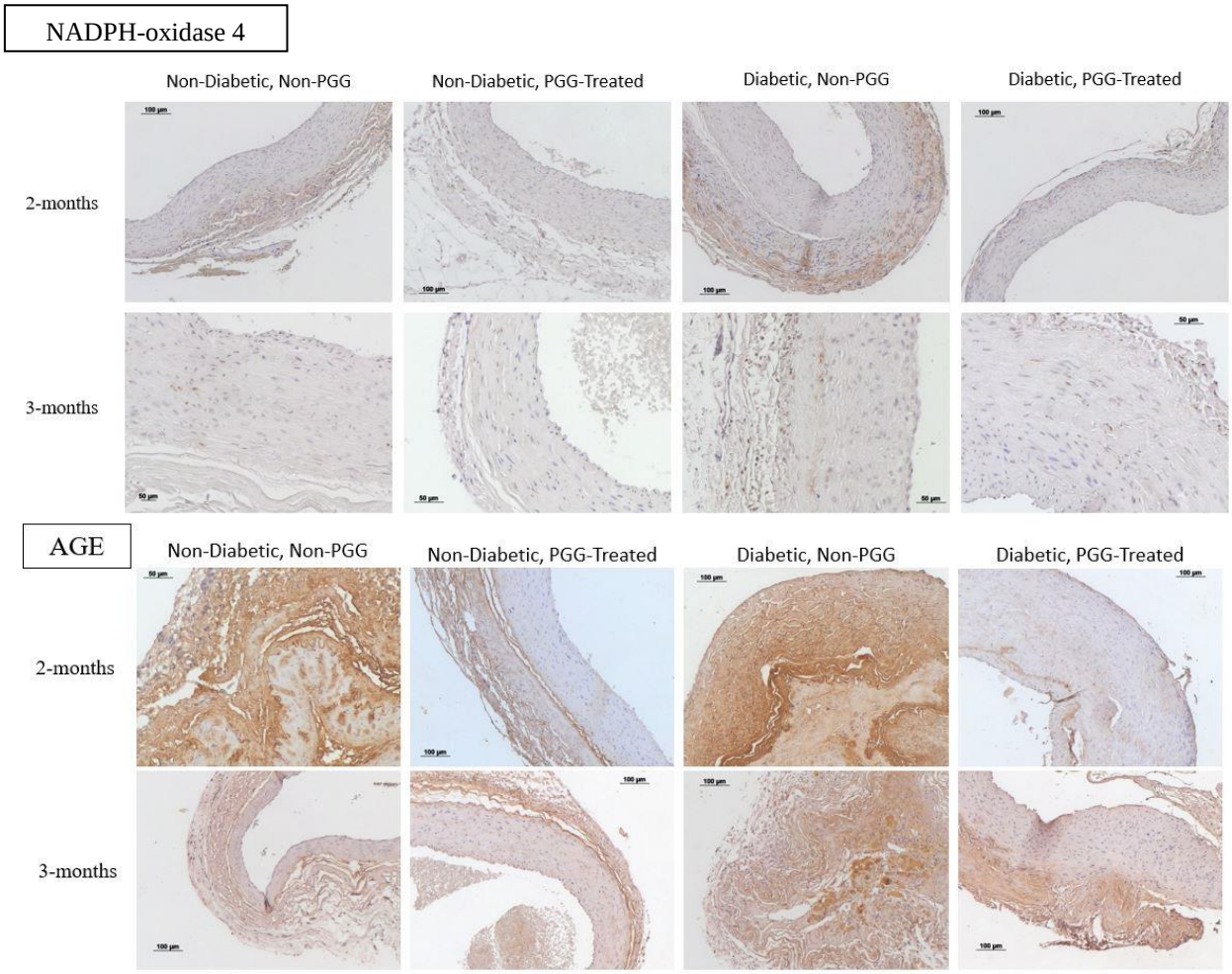

Fig. 4.2.5. Immunohistochemistry for NADPH-oxidase 4 and AGE expression. Expression of NADPH-oxidase 4 and AGE is showed at both 2 and 3-month time-points (n=6). Brown=positive expression of markers. Images were taken at 100X and 200X.

4.4. Discussion

ECM based scaffolds have been extensively researched and used in many tissue engineering and regenerative medicine applications, for both preclinical animal studies and clinical applications in humans.[4,5] These scaffolds have shown to facilitate constructive remodeling and site appropriate tissue reconstructions, the mechanisms of

which are still not well understood. The 3-D architecture of the ECM components are uniquely suited to the tissue from which the ECM is derived, therefore, the scaffolds consist of the structural and functional molecules secreted by the resident cells of the tissue and organ from which they are harvested. The mechanical behavior and cellular infiltration patterns of ECM scaffolds change during *in vivo* remodeling and these changes are dependent on factors such as local tissue microenvironment, rate of scaffold degradation, mechanical forces present, and the rate and extent to which the infiltrating cells deposit new ECM.[6] The ECM also influences the behavior and phenotype of the infiltrating cells, and cell attachment, migration, proliferation and 3-D spatial arrangement is highly affected by matrix composition and structure.[4]

Alterations in ECM accumulation, composition and organization can adversely affect tissue function. One of the most detrimental environments that can alter the ECM and affect its proper remodeling and interaction with cells is the onset of diabetes. During the progression of diabetes, changes in ECM protein expression lead to structural modifications of the ECM network and altered cell-ECM and cell-cell interactions. Specifically in vascular tissues, diabetes-induced alterations to the vascular ECM are associated with several complications.[7] Collagen and elastin-based scaffolds implanted sub-dermally in diabetic rats revealed significant structural alterations caused by the formation of AGEs.[3] If implanted in diabetic patients, these scaffolds would be severely modified by the high glucose and lipid concentrations.[2] The consequences of severe matrix alterations in diabetes include impaired healing, remodeling, and tissue

regeneration; all of which are key processes highlighted in tissue engineering research and applications.

In the studies presented here, we show that ECM-based scaffolds for blood vessel tissue engineering were affected by diabetes when implanted in experimentally induced diabetic rats. Acellular porcine renal artery scaffolds implanted at the abdominal aorta position of diabetic rats showed modifications in elastin structure, high levels of inflammatory cell infiltration, and increased AGE accumulation and oxidative stress when compared to scaffolds implanted in non-diabetic rats. Based on previous research, PGG-treatment of scaffolds demonstrated excellent resistance in that the treated scaffolds showed reduced inflammatory cell infiltration, reduced accumulation of AGEs and oxidative stress, and overall better remodeling and integration with the native tissue.[3] These results were obtained from a sub-dermal implantation in a diabetic rat model; moving forward, we wanted to determine whether we could show similar findings when the scaffolds were implanted as vascular grafts as functional blood vessels.

Degradation of an ECM scaffold is inevitable and necessary, to some degree for the eventual success of the scaffold. The concept of constructive remodeling refers to the process by which the scaffold is degraded and gradually replaced by anatomically appropriate and functional tissue. Remodeling can also consist of chronic inflammation when the ECM structure and composition is altered,[8] under conditions such as diabetes, pro-inflammatory remodeling is accelerated. Therefore, for tissue engineered blood vessels for diabetic patients, the process of degradation and remodeling must be gradual. Upon implantation, the grafts are expected to experience the repetitive, physiological

forces and loads of aortic blood flow. To survive this in a diabetic environment, we incorporated penta-galloyl glucose (PGG) as a treatment to hinder degradation of the scaffold. Several papers have shown the benefits of PGG-treatment, especially its ability to reduce degradation and stabilize elastin.[3,9–12] It has been shown that PGG has a strong binding affinity to aortic elastin and, in this manner, renders elastin highly resistant to degradation. Local delivery of PGG to rat abdominal aorta prevented aneurysm formation and also showed that PGG is not toxic at local or systemic levels.[10,13,14] The anti-oxidant capacity of PGG has been evaluated in several models, and it has demonstrated a remarkable ability to scavenge free radicals even better than vitamin E. For these studies, we hypothesized that by delaying host tissue remodeling processes with PGG-treatment, we would maintain vascular graft structural integrity and patency for a longer period of time and allow for gradual, reparative remodeling to occur.

To test this hypothesis in an *in vivo,* circulatory model, Sprague-Dawley rats were chosen as the host species for implantation of the vascular scaffolds. This model was selected due to practical reasons such as minimal cost, ease of maintenance, and ease of inducing diabetes. Small rodents are the most widely used preclinical animal model to study metabolic disorders such as diabetes. Furthermore, their physiology is closer to that of humans so the results seen could be applicable to what would occur in a human model.[15] Streptozotocin (STZ) was selected as the method for inducing diabetes because it is a fast and guaranteed approach; 72 hours after administration, the rats had achieved diabetic levels of glucose. Alloxan is another diabetogenic drug that can be used to

induce diabetes in animal models, however, STZ was chosen because it is more stable and less toxic.[15,16]

During the implantation period, we wanted to observe the status of the grafts at different time points to patency and blood flow through the grafts. Ultrasound imaging allowed us to do so, specifically using color and pulse wave Doppler imaging. This imaging modality uses the Doppler effect, which is essentially shift in sound wave frequency that occurs when a sound wave is reflected by a moving target (blood cells,) to determine blood flow velocity and direction, allowing us to determine whether the graft was patent or not. If the blood cells are flowing toward the transducer, the frequency of the reflected waves is higher than the original transmitted waves. This difference in transmitted and reflected wave frequency is the "Doppler shift." The greater the Doppler shift, the greater the velocity of the blood flow.[17] We imaged the abdominal aorta region of the rats at four different time points: before implanting the scaffolds, right after implantation, mid-way through the implantation period, and just before explanting the scaffolds. Doing so helped us approximate the lifespan of the grafts, and whether blood flow and patency changed throughout the implantation period. By the end of the 2-month time point, almost 63% of the PGG-treated grafts remained patent (5 out of 8) and only 36% of the non-treated grafts remained patent (4 out of 11). Of the diabetic rats, approximately 67% of the PGG-treated grafts were patent (4 out of 6) and only 33% of the non-diabetic grafts remained patent (2 out of 6). This was an important preliminary indicator of the improved ability of PGG-treated grafts to withstand diabetic conditions compared to the non-treated grafts. By the end of the 3-month time point, the

comparison between the PGG-treated and non-treated grafts became less apparent, as both groups had a 75% patency rate. Only one graft from the diabetic group was non-patent in both graft types while all of the non-diabetic grafts remained patent. Patency is one of the most important indicators of vascular graft success, because low patency is a sign of intimal hyperplasia and occlusion.[18] The ability of the PGG-treated grafts to remain patent under diabetic conditions is very promising, since it suggests that they may have improved patency rates compared to the decreased 5-year patency rate of synthetic grafts such as Dacron and ePTFE in small-diameter applications.[19,20]

At the time of explantation, the non-patent grafts were noticeably more difficult to retrieve. Macroscopically, they were visibly degraded and occluded and had, in some cases, shrunken in size. The patent grafts, in contrast, were easy to recover and maintained their shape and structure at both the 2 and 3-month time points. To gain a better understanding of the integrity and modifications of the ECM after each time point, each sample was examined histologically. As seen in **Figure 4.1.8**, the overall ECM did not appear to be significantly altered and seemed to integrate well with the native abdominal aorta. H&E showed excellent preservation of the overall ECM microstructure and plenty of host cell infiltration. Overall, H&E did not show much differences between the PGG-treated and non-treated grafts at both time points. Differences were apparent, however, from the VVG Trichrome staining of the 3-month grafts. All of the grafts, PGG-treated and non-treated, showed decent preservation of elastin at the 2-month time point. Although the non-treated grafts shown are non-patent, the elastin fibers appear to remain intact and well-preserved. At 3 months, the PGG-treated grafts showed better

elastin preservation than the non-treated grafts, which showed diminished elastin integrity. This is an important consideration because degradation of elastin in cardiovascular tissues has shown to encourage calcification at these sites and promote development of vascular disease.[12,21,22] While we did not find evidence of calcification (not shown), it is likely in a different model, possibly one with a longer implantation period, would have some calcification in the non-treated groups.

To determine the ability of the grafts to integrate with the native abdominal aorta and initiate the process of remodeling, we look for infiltration of host vascular cells. As shown in **Figure 4.1.9**, the grafts from each group successfully formed a uniform endothelial layer in the intima as well as a dense, uniform smooth muscle cell layer in the media. Overall vascular cell infiltration showed no differences between the treatment groups or time points. Therefore, it was determined that the decellularized, acellular scaffolds encourage infiltration of vascular cells and that PGG-treatment does not inhibit infiltration of appropriate cells after implantation. This was the first indication of a normal host response and constructive remodeling.

Once it had been established that basic vascular cell infiltration had occurred, the next aspect of the implant-host response to evaluate was the overall immune and inflammatory response. The immune response of a host to the implant is a major indicator of biocompatibility. Biocompatibility is vaguely defined as "the ability of a material to perform with an appropriate host response in a specific situation."[23] At the implant site of any tissue engineered construct, the scaffold is subjected to inflammatory mediators and signaling molecules such as cytokines, growth factors, and ECM enzymes

and proteins which is different than the native environment of the tissue from where the scaffold was derived. Depending on the scaffold type, these factors can evoke various responses such as cellular activation, differentiation, proliferation or migration.[24] In our study, we did not see an extreme density of invading inflammatory cells in the scaffolds. Overall, we did not see any indications of an aggressive inflammatory response from the host nor did we see any presence of foreign body giant cells. To determine this, we looked at the presence of T-cells and macrophages in the scaffolds. Both of these cell types play an important role in the inflammatory response. Lymphocytes such as T-cells have been observed to associate with macrophages and foreign body giant cells. It was demonstrated that lymphocytes enhanced macrophage adhesion and fusion while macrophages stimulated lymphocytes to proliferate.[24] To learn whether PGG treatment had an effect on T-cell recruitment, we look for CD8 expression, a marker for T-cells. As seen in **Figure 4.2.1**, at both time points, the PGG-treated grafts consistently showed lower CD8 expression in the grafts. There was not a significant difference between the diabetic and non-diabetic PGG-treated grafts at the 2-month time point, but at the 3-month time point, the diabetic PGG-treated graft did show higher expression of CD8 than the non-diabetic PGG-treated graft. The non-treated grafts showed significantly higher expression of CD8, and the non-treated scaffolds in the diabetic rats showed the highest CD8 expression at both time points. It is very possible that due to PGG treatment and the resistance it provides scaffolds to degradation, the host was faced with less cytokines that would evoke an inflammatory response. Due to the stability of the treated scaffolds, T-cell recruitment was discouraged. Moving forward, to examine the presence of

macrophages, we looked for CD68 expression, a pan macrophage marker (**Figure 4.2.2).** All groups showed significant infiltration of macrophages. Interestingly, the diabetic PGG-treated grafts had the highest macrophage expression at both 2 and 3-month time points. There were no significant differences between the treated and non-treated grafts in the non-diabetic rats. As discussed earlier, there are differing macrophage phenotypes, therefore, the ratio between M1 to M2 macrophages is more important to identify.

Macrophages play a critical role in inflammation and in the success of ECM scaffold remodeling.[25] In many cases, macrophages are considered to be a beneficial component of the innate immune system, as a modulator of disease and tissue remodeling. Macrophages, due to their remarkable functional plasticity, play both positive and negative roles in the process of tissue remodeling.[26] Upon migration into sites of inflammation, macrophages become activated in response to signals present in damaged tissue. In response to environmental cues, macrophages become polarized and acquire either an M1 or an M2 phenotype. M1, or "classically activated" macrophages express ROS, pro-inflammatory cytokines such as interleukin (IL)-12, IL-6, and TNF-α, and produce high levels of inducible nitric oxide synthase (iNOS). Conversely, M2, "alternatively activated," macrophages express high levels of IL-10 and transforming growth factor beta expression (TGF-β), produce arginase, inhibit the release of pro-inflammatory cytokines, scavenge debris, promote angiogenesis, and recruit cells involved in constructive tissue remodeling. Based on these properties, M1 macrophages are characterized as pro-inflammatory, cytotoxic, and are associated with chronic inflammation; M2 macrophages are characterized as anti-inflammatory, and promote

immunoregulation, tissue repair, and constructive tissue remodeling.[25–27] Since it is known that M1 macrophages express iNOS, and M2 macrophages express CD163 in rats[26], we looked for these markers in our explanted grafts. As seen in **Figures 4.2.3** and **4.2.4**, the diabetic non-treated grafts expressed the highest amount of iNOS at both time points. In contrast, CD163 was expressed in higher quantities in the diabetic PGG-treated grafts, also at both time points. This indicates that even in the diabetic conditions, PGG-treated scaffolds promoted more infiltration of M2 macrophages while the non-treated scaffolds had more M2 macrophage migration. At 2 months, the PGG-treated grafts from both diabetic and non-diabetic rats did not show much iNOS expression. However, by 3 months, the PGG-treated grafts from the diabetic rats did show an increased expression of iNOS but it was still noticeably lower than the diabetic non-treated grafts. Therefore, it is apparent that M2 macrophages are expressed in greater quantities in PGG-treated scaffolds in both diabetic and non-diabetic conditions and iNOS expression is greater in diabetic grafts that are not treated. As mentioned before, the pan-macrophage expression (CD68) was higher in the PGG-treated grafts and was not significantly different between the treated and non-treated groups. However, it does appear that the ratios of M1 and M2 macrophages between the PGG-treated and non-treated groups was significantly different. PGG's ability to encourage the M2 phenotype and inhibit the M1 phenotype is a critical factor for the success of this scaffold for a vascular graft application in diabetes. By inhibiting the pro-inflammatory M1 macrophages, further degradation of the scaffold was prevented.

The primary culprits for vascular disease progression in diabetes are AGEs and oxidative stress. AGE accumulation in the vascular walls mediates the process of inflammation and oxidative stress, along with increased glycation of low-density and high-density lipoproteins (LDL, HDL), activation of iNOS, and decreased nitric oxide (NO) availability. Studies in animal models and in vitro have shown the impact of AGEs on the function of different cell types involved in atherosclerosis: endothelial cells, platelets, monocytes/macrophages, and vascular smooth muscle cells. Binding of AGEs to receptors for AGEs (RAGE) on the surface of monocytes induces the production of IL-1, TNF-α, platelet-derived growth factor and insulin-like growth factor, which all promote a high inflammatory response.[28] Zhang et. al found that pigs with STZ-induced diabetes had increased NADPH oxidase activity and oxidative stress, resulting in inflammatory responses in porcine coronary artery media and adventitia. After examining the underlying mechanisms in isolated fibroblasts from the coronary artery, they found that AGEs, rather than glucose itself, upregulated the expression of IL-6, VCAM-1, and monocyte chemotactic protein-1 mRNAs, which all contribute to increased oxidative stress.[28,29] Oxidative stress occurs when ROS overproduction exceeds the buffering capacity of antioxidant enzymes and antioxidants, resulting in an imbalance between ROS production and destruction. This tips the balance toward a more oxidative state. The main sources of ROS production in vascular cells include the mitochondrial electron transport system, xanthine oxidase, NADPH oxidases and NOS.[30] To examine the antioxidant properties of PGG and whether it would inhibit oxidative stress in the diabetic grafts, we looked for AGE and NADPH oxidase 4 expression. As

expected, **Figure 4.2.5** shows that the diabetic, non-treated grafts had significant AGE accumulation at both 2 and 3-month time points. In contrast, the PGG-treated grafts from the diabetic rats showed markedly reduced AGE expression in the 2-month time point. At 3 months, AGE expression was slightly increased in the diabetic PGG grafts, but it was still noticeably lower than the expression in diabetic, non-treated grafts. Overall, it was apparent that PGG treatment inhibited AGE accumulation in both diabetic and non-diabetic tissues at both time points. To see how this affected oxidative stress in the grafts, we looked for NADPH-oxidase 4 expression. NADPH oxidases play a central role in ROS production, as they activate other ROS producing enzymes such as NOS, eNOS and xanthine oxidase. NADPH oxidase-4 (NOX4) has specifically been identified in vascular walls, particularly VSMCs, fibroblasts, and endothelial cells.[31] **Figure 4.2.5** shows that the diabetic non-treated grafts had the highest expression of NOX4 at both time points. The diabetic PGG-treated grafts, however, had a much lower expression of NOX4, especially at the 2-month time point. Like AGE expression, NOX4 expression slightly increased at the 3-month time point in the diabetic PGG-treated grafts but was still lower compared to the diabetic non-treated graft at 3-months. There was very few NOX4 positive cells visible in the non-diabetic grafts from both the treated and non-treated groups, suggesting that non-diabetic tissues do not develop significant amounts of oxidative stress. This study makes it apparent that PGG-treatment does in fact, inhibit development of oxidative stress even in diabetic conditions. As discussed in a number of publications, AGE accumulation and ROS can be eliminated by antioxidant

mechanisms[11,32–34] and we clearly observe this in these studies by the incorporation of PGG-treatment in our scaffolds.

4.5. Conclusion

The success or failure of an implanted tissue engineered scaffold is entirely based on the host response and biocompatibility of the scaffold. As research into completely biological, tissue-based biomaterials progresses even further, it is very important to consider not only the basic host response to the material, but also how a disease state such as diabetes will affect the overall host response and long-term outcome of the implant. ECM-based scaffolds are an excellent source to build the foundation for developing tissue engineered vascular grafts, due to the familiarity of the proteins that make up the scaffold to the native host environment and its ability to incorporate cells to eventually transform into completely regenerated tissue. Since diabetic patients make up a significant portion of the population requiring revascularization procedures, it is imperative that any tissue engineered vascular scaffold should be able to withstand the harsh diabetic environment and continue to function properly while resisting diabetes-related modifications.

The studies discussed above were all attributed to the scaffold developed in chapter 3. Our aim was to better understand how PGG-treated scaffolds would function in a circulatory animal model of diabetes and whether these scaffolds would be able to resist the inflammation and oxidative stress generated by hyperglycemia and consequent AGE accumulation. We implanted the scaffolds for 2 different time points, 2 and 3-months, to see whether extended time would have any effect on the lifespan of the

scaffolds. We focused our studies on the degradation of scaffolds, overall ECM quality, the inflammatory response including identification and polarization of macrophages, and AGE accumulation and oxidative stress.

The results led us to conclude that PGG-treatment of our scaffolds provided many benefits to the scaffolds. We already knew that PGG significantly decreased degradation, calcification, inflammation, AGEs, and oxidative stress as a subdermal implant. However, we wanted to see these results replicated with these PGG-treated scaffolds were implanted as vascular grafts at the abdominal aorta position of rats. Remarkably, we found that the PGG-treated grafts resisted elastin degradation, inflammation, and AGEs and oxidative stress, just as they did sub-dermally. It is evident that treatment with PGG provides significant protection to tissue engineered scaffolds intended for implantation in a diabetic environment and will allow for a more gradual remodeling and integration with the host vasculature.

4.6 References

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CHAPTER 5

ASSESSING THE EFFECT OF STEM CELL DIFFERENTIATION AND SEEDING ON SCAFFOLD REMODELING IN A DIABETIC ENVIRONMENT

5.1 Introduction

The incorporation of stem cells in the development of our TEVGs was a design consideration of significant interest. We chose to focus on adipose tissue as the stem cell source to seed the scaffolds. We selected adipose derived stem cells as our source for three main reasons: they are easy to harvest in abundant quantities, they have demonstrated the ability to differentiate into endothelial cells and smooth muscle cells, and they also have immunomodulatory properties which is advantageous for scaffold remodeling. Previously, it was observed that ADSC-seeded scaffolds implanted sub-dermally in diabetic rats exhibited reduced accumulation of T-cells and pan-macrophages, and increased presence of the pro-remodeling M2 macrophages compared to the pro-inflammatory M1 macrophages.[1] For this research, we decided to treat the ADSCs with specific growth factors to promote differentiation into ECs, SMCs, and fibroblasts, to test the differentiation potential of ADSCs.

We hypothesize that implanting differentiated ADCS-seeded vascular scaffolds in diabetic rats will exhibit similar results as the previous studies, in that the cell-seeded grafts will show reduced inflammatory cell infiltration and an improved remodeling and integration response compared with the acellular implanted scaffolds in diabetic conditions. To test this hypothesis, we isolated ADSCs from Sprague-Dawley rats, divided them into three groups, and treated each group with growth factors vascular endothelial growth factor (VEFG) and insulin-like growth factor (IGF-1) to promote EC

differentiation, transforming growth factor beta (TGF-β) and bone morphogenic protein 4 (BMP-4) for SMC differentiation, and TGF-β for differentiation into fibroblasts. After cells had proliferated to sufficient quantities, they were statically injected into the scaffolds. The cell-seeded scaffolds were implanted as vascular grafts into Sprague-Dawley rats for a period of 16-weeks, and the rats were induced with diabetes. Ultrasound imaging was performed to monitor blood flow and graft patency. After explanting, the grafts were characterized for host cell infiltration, inflammatory response, and overall remodeling using histological and protein analysis. Cell differentiation was determined using immunofluorescence to identify specific cell markers to indicate differentiation into vascular cells.

5.2. Materials and Methods

5.2.1. Adipose Stem Cell Isolation and Differentiation

Adult male Sprague-Dawley rats (n=25) were ordered from Charles River Laboratory and allowed 7 days' time to acclimate. The rats were prepped for surgery and placed under anesthesia (2-3% Isoflurane). A small amount of subcutaneous abdominal fat (~30mg) was harvested from each rat via lipectomy and immediate processed to isolate adipose stem cells (ASCs). Briefly, the adipose tissue was minced, incubated in collagenase, washed with ammonium chloride to remove red blood cells and centrifuged. The stromal vascular fraction pellet was plated in tissue culture flasks and cultured for 2 weeks to propagate the adipose stem cells.

After the ASCs had reached confluency, they were prepared for differentiation into endothelial cells, smooth muscle cells and fibroblasts. The cells from each animal

were maintained separately in labeled flasks to allow for autologous re-implantations. Each animal's cells were split into 3 separate flasks, to be treated with the specific combination of growth factors pertaining to the cell type. For endothelial cells, VEGF at 0.5 ng/mL and IGF-1 at 20 ng/ml were added; for smooth muscle cells, TGF-β at 5 ng/mL and BMP-4 at 2.5 ng/mL were added; and for fibroblasts, TGF-β at 2 ng/mL was added. All growth factors were ordered from Peprotech. These growth factors were added to the cell media during each media change and cells were maintained at 80% confluency. The cells were treated with the growth factors for 2 weeks to differentiate the cells. Immunofluorescence (IF) was performed to identify specific cell markers pertaining to the target cell type which determined whether differentiation was successful.

5.2.2. Immunofluorescence for Differentiated ADSCs

Prior to IF, the cells were plated in a 6 well plate. To perform IF, the media was aspirated from the wells and the cells were rinsed with warm 1X PBS. Next, the cells were fixed with 4% warm paraformaldehyde for 30 minutes. The cells were rinsed three times with 1X PBS, 5 minutes per wash. Then, they were permeabilized with 0.2% Triton in 1X PBS for 10 minutes. The next step was blocking with the blocking agent (BA), which was prepared with 0.2% Triton, PBS, and Bovine Serum Albumin (BSA), for 2 hours. The primary antibodies applied were anti-vWF (Abcam 6994, 1:400 dilution), anti-CD31 (Abcam 250589, 1:500 dilution) for endothelial cell identification, anti-SM22-α (Abcam 14106, 2.5ug/ml dilution), anti-Actin (Abcam 3280), anti-SMH-Myosin (Abcam 683, 1:250 dilution) for smooth muscle cells, anti-Vimentin (Abcam

92547, 1:500 dilution), and anti-Hsp47 (Abcam 77609, 1 µg/mL dilution) for fibroblast identification, were prepared in a 1:1 BA and 1X PBS solution and added to the wells. The cells were incubated with the primary solution for a minimum of 2 hours at room temperature on a shaker. After primary incubation, the cells were rinsed with PBS three times for 5 minutes per wash. The secondary antibody, Alexa Fluor 488 (Life Technologies), was diluted at 4 µg/ml in BA and applied for 1 hour at room temperature in the dark. After secondary incubation, the wells were replaced with DAPI solution at a concentration of 1 µg/mL for 5 minutes. The cells were rinsed one last time in 1X PBS and imaged via fluorescence microscopy immediately.

5.2.3. Seeding of Differentiated Cells into Scaffolds

The differentiated cells were passaged and resuspended in 250 µL of media (fibroblasts were resuspended in 1 mL of media). The cells were then seeded into each artery scaffold (Obtained from the same procedures described in Chapter 3) at approximately $1X10^6$ cells per scaffold via injection with 30G needle. The scaffolds for this study were not treated with PGG. First, the SMCs were injected directly into the media layer of the artery at multiple sites along the scaffold; then, one end of the artery was ligated with a suture and the ECs were injected into the lumen and then the other end of the artery was ligated. Finally, the scaffold was placed in a well plate with the fibroblast suspension surrounding the scaffold to seed the adventitia. The re-cellularized scaffolds were placed in the incubator overnight prior to implantation. A few cell-seeded scaffolds were fixed in 10% neutral buffered formalin and paraffin-embedded. The

scaffold sections (5 μ) were rehydrated and stained with hematoxylin and eosin (Richard-Allen Scientific, Thermo Scientific) to confirm presence of ASCs.

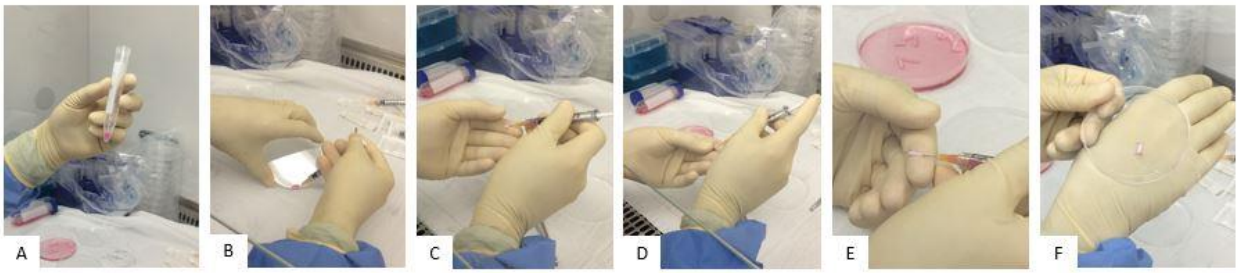


Fig. 5.1.1. Scaffold cell seeding procedure: (A) Differentiated cells in suspension. (B) Cells collected in syringe. (C and D) SMCs injected in the media. (E) One end of the scaffold was ligated, and ECs were injected into the lumen. (F) Scaffold placed in a well plate containing FB suspension.

5.2.4. Implantation of Cell-Seeded Vascular Grafts (Study 3)

Adult male Sprague-Dawley rats (n=25, weight 300-350 g, Charles River) were ordered and allowed 10 days' time to acclimate. After the acclimation period, rats were prepped for surgery and anesthetized using 1-2% Isoflurane. The same surgical procedure previously described (Aim 2) was used to implant the cell seeded vascular grafts. Half of the rats were induced with diabetes using the STZ administration procedure described. After 12 weeks, the rats were humanely euthanized by CO_2 asphyxiation and the vascular grafts were explanted and collected according to their respective application as follows: grafts for histological analysis were placed in 10% phosphate-buffered formalin and paraffin embedded; and samples for protein analysis were flash frozen in liquid nitrogen and kept on dry ice until transferred to -80℃ for storage.

	Rat Number	
DIABETIC	5	acellular
	8	acellular
	12	acellular
	17	cell-seeded
	19	cell-seeded
	20	cell-seeded
	22	cell-seeded
NON-DIABETIC	2	cell-seeded
	7	cell-seeded
	13	cell-seeded
	15	cell-seeded
	16	cell-seeded
	18	cell-seeded
	24	acellular

Table 5.1. Experimental implant groups for study 3: 3 diabetic acellular grafts, 4 diabetic cell-seeded grafts, 6 non-diabetic cell-seeded grafts, and 1 non-diabetic acellular grafts

5.2.5. Ultrasound Imaging

Ultrasound imaging was performed using the same procedure described previously (Chapter 4). The rats were imaged at four time points: before surgery, post-surgery, mid-way into implant period, and before explanting.

5.2.6. Histological Analysis

Paraffin sections (5 µm) were rehydrated and stained with Hematoxylin and Eosin (H&E) for a general analysis of ECM morphology and host cell infiltration during implantation. Masson's Trichrome and VVG staining was used to further assess ECM composition and collagen and elastin integrity.

Immunohistochemistry (IHC) was performed for detection of various cellular markers in the explanted grafts. The IHC procedure described previously was used. The

results were compared to the native abdominal aorta as well as analyzed for differences between diabetic and non-diabetic and cell-seeded and acellular grafts. Digital images were obtained at various magnifications (25X-100X) on a Zeiss Axiovert 40CFL microscope using AxioVision Release 4.6.3 digital imaging software. The following antibody dilutions and concentrations were used: vWF (1:1000), α-smooth muscle actin (1:500), CD8 (1:100), CD68 (1:100), CD163 (1:500), iNOS (1:100), AGE (1:1000), and NADPH-oxidase (1:500).

5.3 Results

5.3.1. Diabetes and Weight Monitoring in Rats

As seen in the previously conducted study (section 4.3.1), STZ administration successfully induced diabetes in the rats after 72 hours. Glucose levels ranged between 355-645 mg/dL in diabetic rats and 100-145 mg/dL in the control, non-diabetic rats. Diabetic rat weights ranged between 450-480 g and control rat weights between 515-570 g.

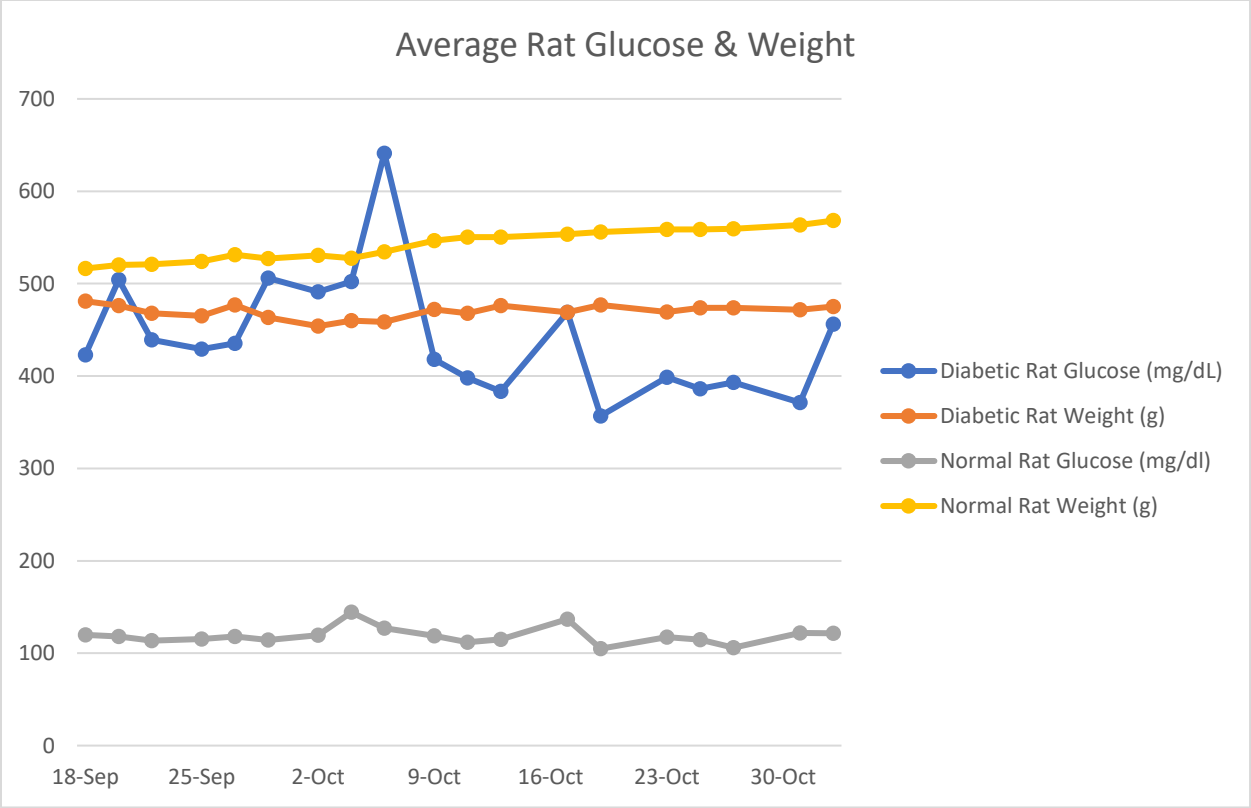


Fig. 5.1.2. Average glucose and weight measurements in diabetic and control rats over a 12-week period

5.3.2. Assessment of Stem Cell Differentiation

For this study, it was decided that instead of injecting ADSCs directly into the scaffolds, we would pre-differentiate the ADSCs using specific growth factors. To verify whether the growth factor treatments resulted differentiation into endothelial cells, smooth muscle cells, and fibroblasts, immunofluorescence was performed for various markers. Endothelial cells, which were treated with IGF and VEGF, were stained for CD31 and vWF. Smooth muscle cells, which were treated with TGF-β and BMP-4, were stained for actin, SM22 and SMH-Myosin. Fibroblasts, which were treated with TGF-β, were stained for actin, vimentin, and Hsp47. As seen in **Fig. 5.1.3**, the

immunofluorescence staining is shown, and cells clearly expressed each marker pertaining to their cell type, indicating that the ADSCs had successfully differentiated into the target vascular cell types.

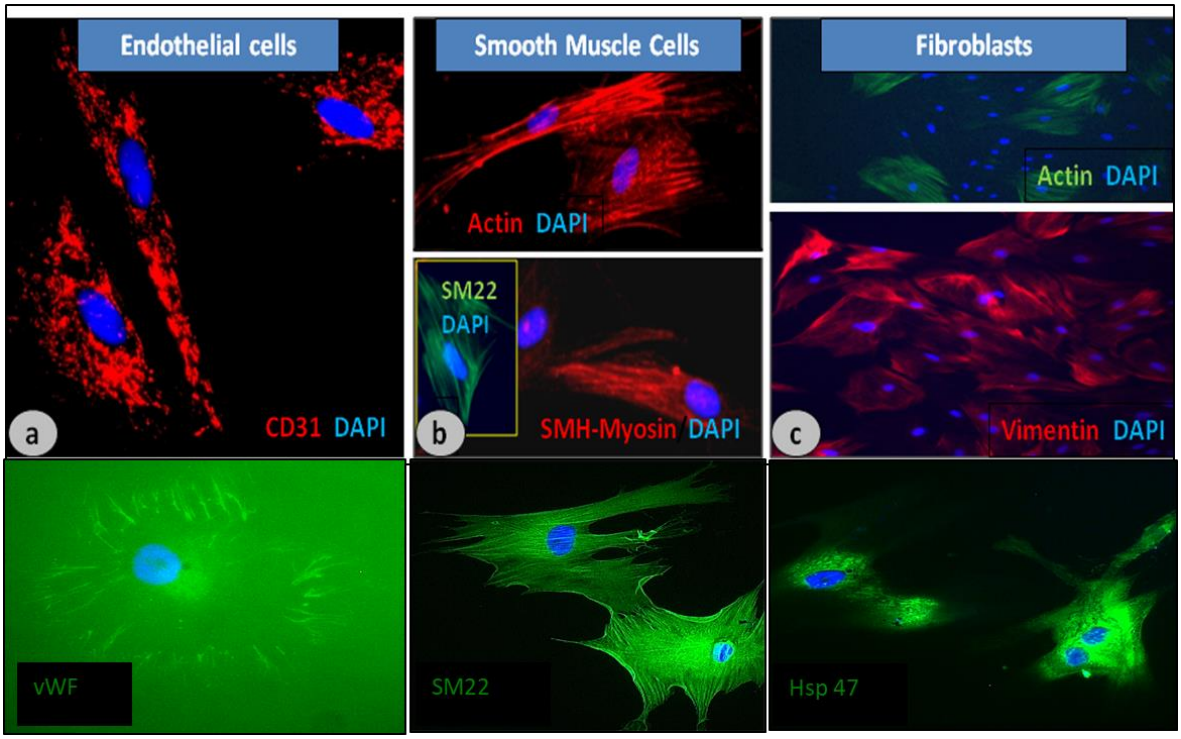
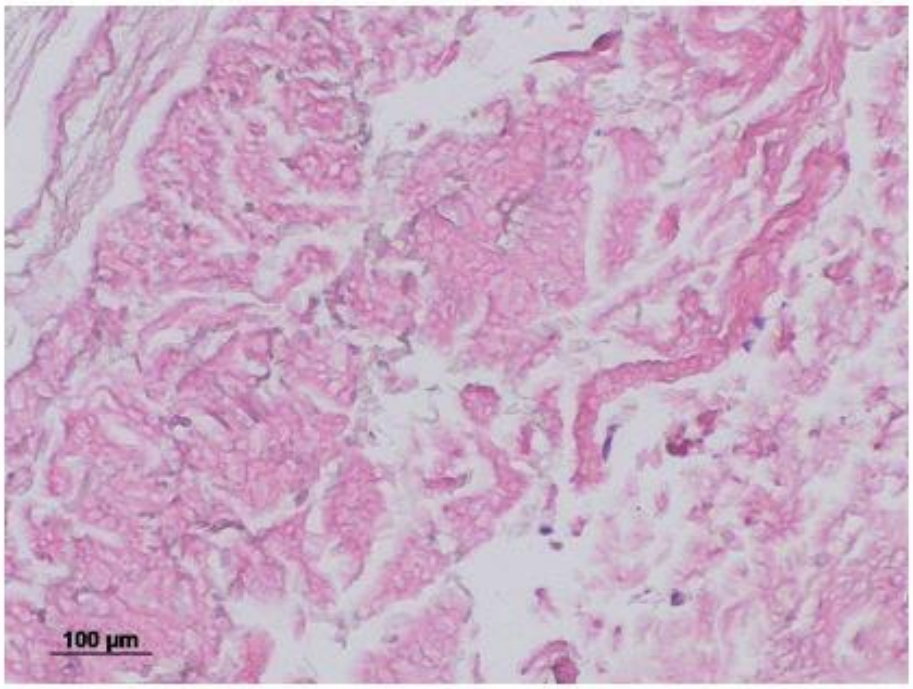


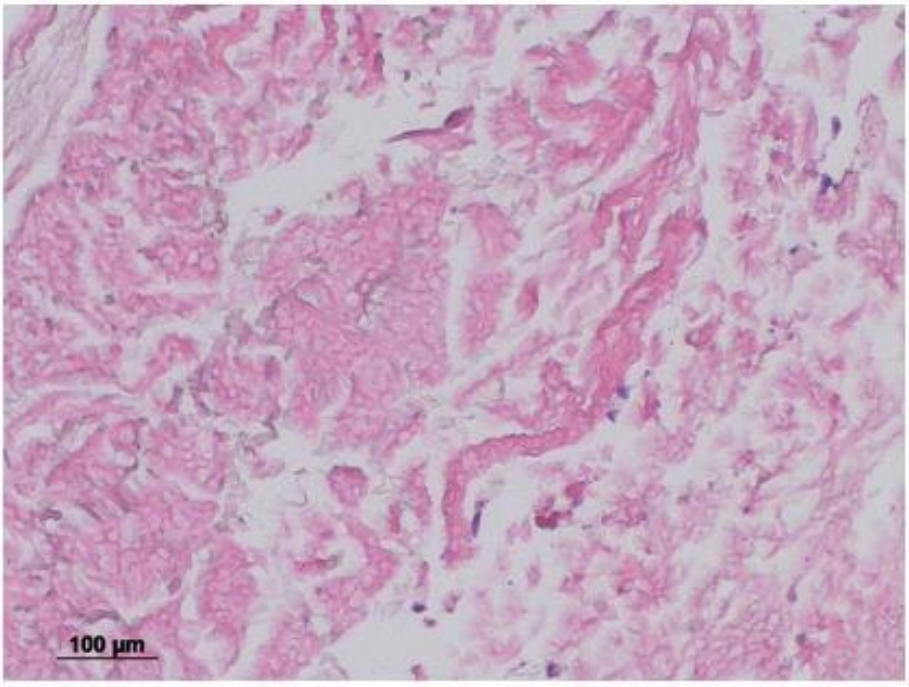
Fig. 5.1.3. Immunofluorescence staining of pre-differentiated ADSCs to identify specific markers to confirm differentiation. Endothelial cell markers CD31 and vWF, smooth muscle cell markers actin, SMH-Myosin, and SM22, and fibroblast cell markers actin, vimentin and Hsp47 were all positively expressed.

5.3.3. Evaluation of Cell Seeding Efficacy

The vascular graft scaffolds were statically seeded with the pre-differentiated ADSCs one day before implantation and incubated overnight. To verify whether the cells had attached to the scaffolds, samples were processed in 10% neutral-buffered formalin and stained with H&E to detect presence of cell nuclei. Although the presence of cells in the scaffolds was visually sparse, a few cells were observed mostly in the adventitial

layer of the arteries, confirming that some cell attachment had occurred. This can be seen in **Fig. 5.1.4.**





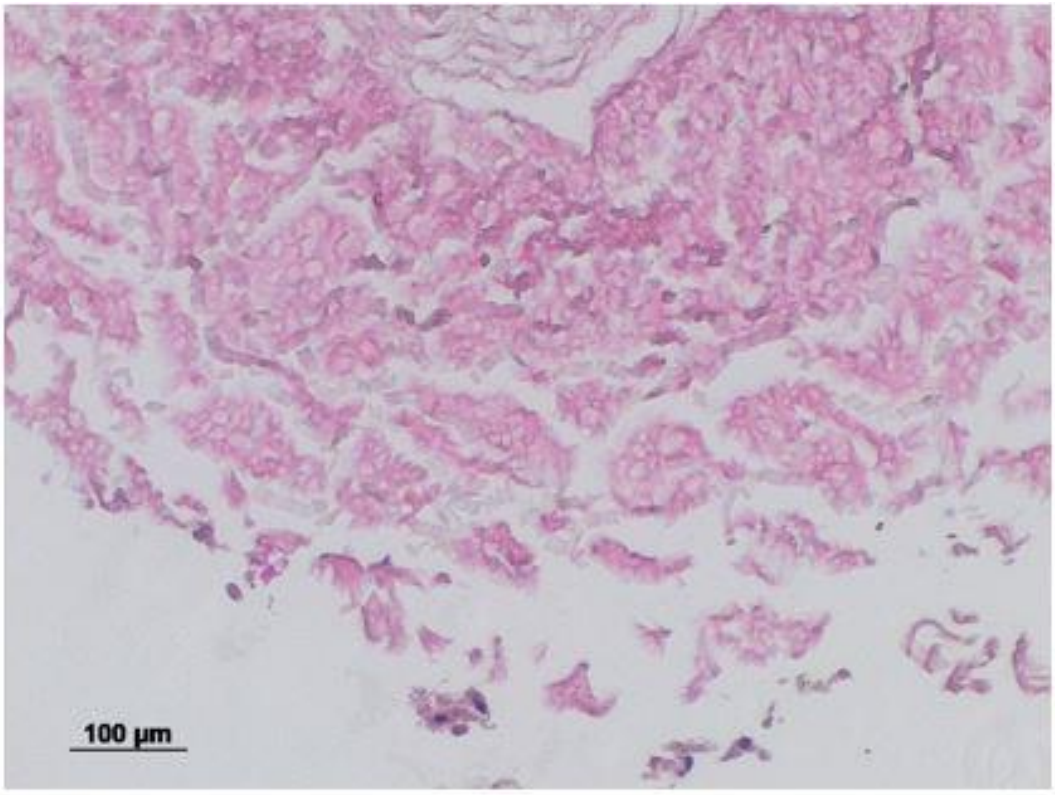


Fig. 5.1.4. Histological images of scaffolds seeded with differentiated ADSCs and stained with Hematoxylin & Eosin. Blue=cell nuclei, pink=matrix components. Images were taken at 100X.

5.3.4. Assessment of Graft Patency with Ultrasound Imaging

Ultrasound imaging provided us with the ability to observe the patency and overall status of the implanted vascular grafts as described previously (section 4.3.2). **Table 5.2** indicates the rats whose grafts remained patent and non-patent by the 3-month time point.

	Rat #		Patent	Non-Patent
	17	cell-seeded	X	
DIABETIC	19	cell-seeded		X
	20	cell-seeded	X	
	22	cell-seeded	X	
	13	cell-seeded	X	
	15	cell-seeded		X
NON-DIABETIC	16	cell-seeded	X	
	18	cell-seeded	X	
	24	acellular	X	

Table 5.2. Patent and non-patent grafts from each group as observed by Ultrasound imaging.

5.3.5. Explant Analysis- ECM and Host Cell Infiltration

To assess how the diabetic rats reacted to the implanted cell-seeded grafts, H&E and VVG staining was performed to observe the pattern of host cell infiltration and alterations in the ECM. H&E staining showed significant host cell infiltration in all the grafts. No differences were observed in the overall amount of cell infiltration between the cell-seeded grafts and acellular grafts in the diabetic or non-diabetic environments. VVG staining showed preservation of elastin in all the graft samples, although the diabetic cell-seeded graft did show slight degradation of elastin compared to the non-diabetic grafts. There were no significant differences observed in the preservation and integrity of the elastin fibers between the cell-seeded and acellular grafts.

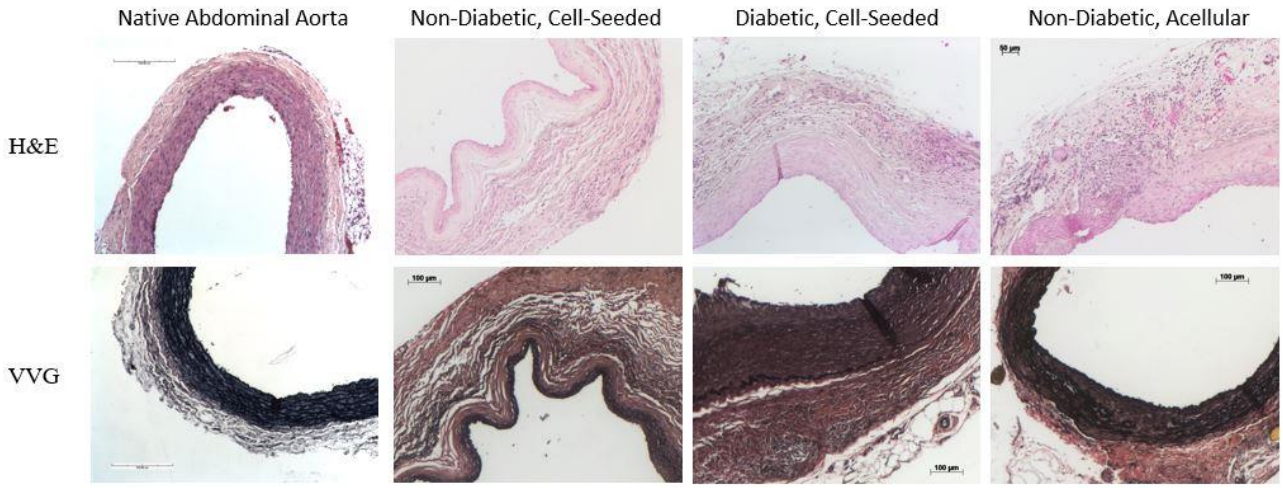


Fig. 5.1.5. Histological images of explanted grafts stained with Hematoxylin & Eosin (H&E) to observe cell infiltration and overall ECM structural integrity and Verhoeff van Gieson (VVG) to assess preservation of elastin. H&E: Blue= cell nuclei, pink= ECM components; VVG: Black=elastin fibers, red/brown= counterstain. Images taken at 100X magnification.

5.3.6. Immunohistochemical Analysis of Vascular Cell Infiltration

To determine whether cell-seeding encouraged the grafts to repopulate with vascular cells, IHC was performed to detect presence of endothelial cells and smooth muscle cells. Staining for vWF showed the formation of a continuous endothelial layer in all of the patent grafts, however, the cell-seeded grafts seemed to show a more defined and uniform endothelium compared to the acellular graft. Staining for α-SMA showed formation of a smooth muscle cell layer in the media in all of the patent grafts, however, the acellular graft did not show as dense and uniform of a smooth muscle layer compared to the cell-seeded grafts. No differences were observed between the diabetic and non-diabetic grafts.

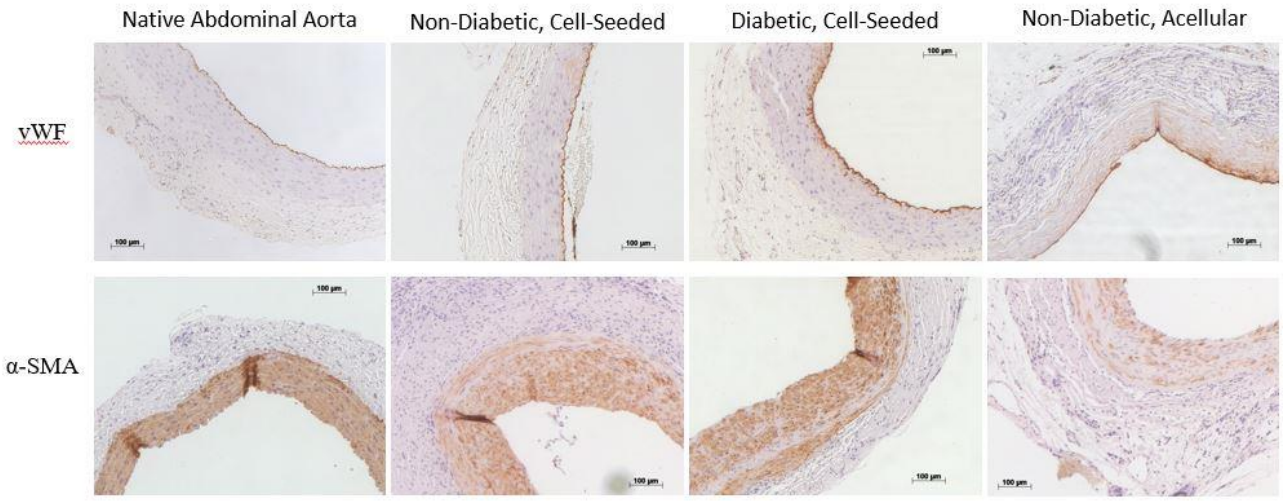


Fig. 5.1.6. Immunohistochemical images of explanted cell-seeded and acellular grafts. The tissues were stained for von Willebrand factor (vWF) for endothelial cells and α-smooth muscle actin for vascular smooth muscle cells. Brown=positive expression of cell marker. Images taken at 100X magnification.

5.3.7. Immunohistochemical Analysis of Inflammatory Cell Infiltration

One of the hypotheses of this study was that the addition of pre-differentiated cells would mitigate the inflammatory response of the host to the implanted grafts. To test this, IHC was performed for the detection of inflammatory cells. The explanted grafts were stained for CD8, CD68, CD163, and iNOS. Staining for CD8 showed that all of the grafts contained T-cells, however, the cell-seeded grafts had fewer T-cells compared to the acellular grafts. The diabetic cell-seeded grafts had slightly more T-cell infiltration compared to the non-diabetic cell-seeded graft, but they still had fewer T-cells than the acellular graft. CD68 staining showed macrophages in all the grafts, and no differences were observed in the number of macrophages between the acellular grafts and the cell-seeded grafts in both diabetic and non-diabetic conditions. To determine whether

the macrophages were of the M1 or M2 phenotype, the grafts were stained with CD163 and iNOS. The cell-seeded grafts showed slightly higher expression of CD163, associated with M2 macrophages compared to the acellular scaffold. There was no significant difference in the number of M2 macrophages in the diabetic cell-seeded grafts compared to the non-diabetic cell-seeded grafts. Very few, if any, iNOS positive cells were present in any of the grafts. The cell-seeded diabetic and non-diabetic grafts had approximately only 1.5% of iNOS positive cells and the acellular grafts had approximately 0.9% of iNOS expression, indicating that by the 3-month time point, M1 macrophages were not significantly present in the grafts.

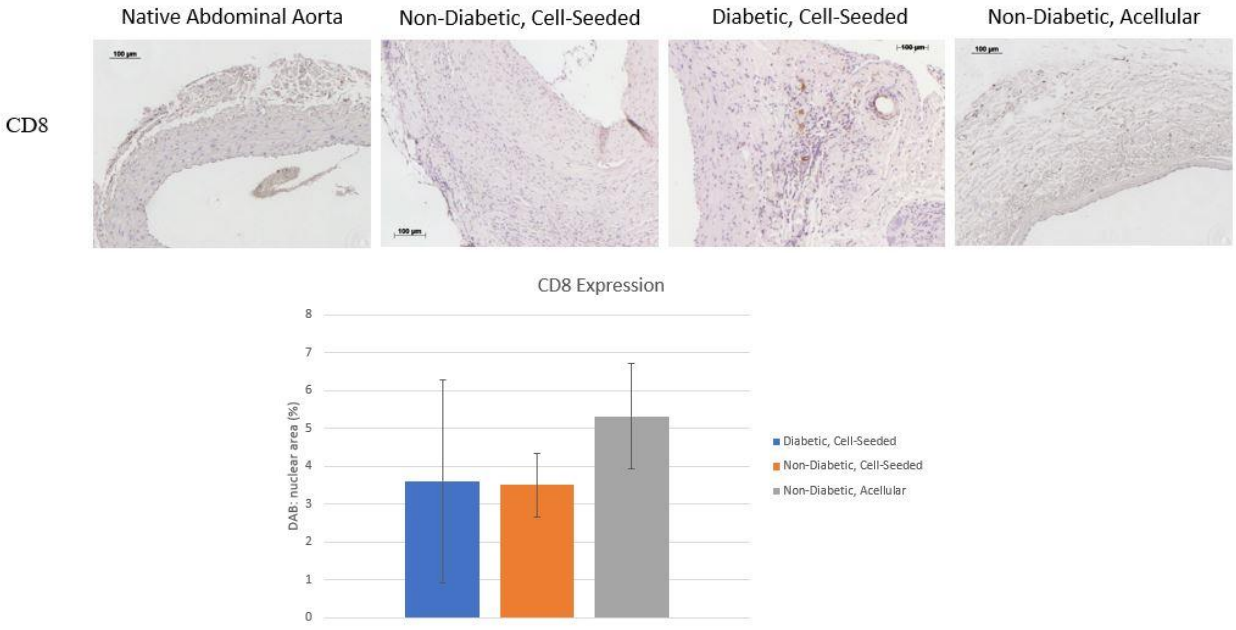


Fig. 5.1.7. Immunohistochemistry and semi-quantification analysis of CD8 expression. CD8 expression in cell-seeded and acellular grafts in diabetic and non-diabetic conditions is shown (n=6). Brown=positive expression of marker. Images were taken at 100X magnification.

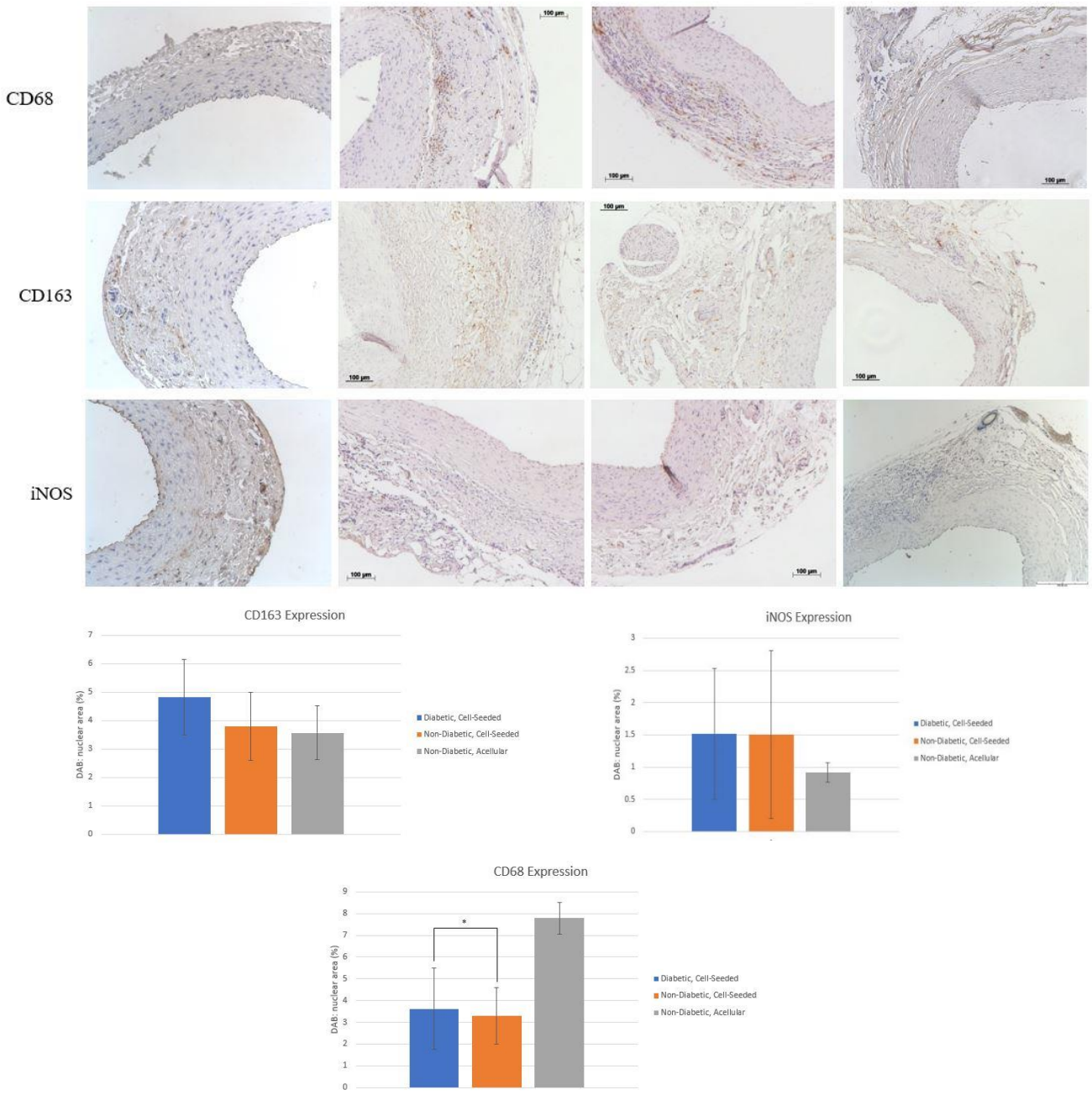


Fig. 5.1.8. Immunohistochemistry and semi-quantification analysis of CD68 (pan-macrophages), CD163 (M2 macrophages) and iNOS (M1 macrophages) expression. Macrophage expression in cell-seeded and acellular grafts in diabetic and non-diabetic conditions is shown (n=6). Brown=positive expression of marker. Images were taken at 100X magnification.

5.3.8 Immunohistochemical Analysis of AGE Accumulation and Oxidative Stress

As described previously, the next step in characterizing the explanted grafts was to observe AGE accumulation and oxidative stress (chapter 4, section 4.3.7); in this study, we wanted to determine whether cell-seeding decreased the formation of AGEs and oxidative stress in the tissues. IHC was performed by staining for AGE and NADPH-oxidase 4. As seen in **Fig. 5.1.8**, the diabetic grafts showed the highest expression of both AGE and NADPH-oxidase 4 compared to the non-diabetic grafts. There was not a significant difference observed between the cell-seeded and acellular grafts in the non-diabetic conditions.

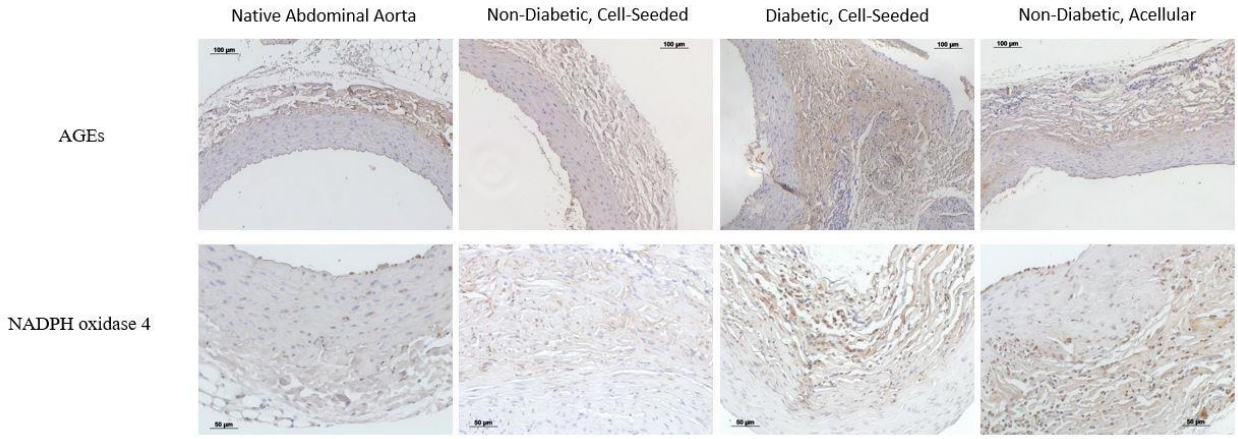


Fig. 5.1.9. Immunohistochemistry analysis of AGE and NADPH-oxidase 4 (oxidative stress) expression. Expression in cell-seeded and acellular in diabetic and non-diabetic conditions is shown. Brown=positive expression. Images were taken at 100X (top panel) and 200X (bottom panel).

5.4. Discussions

Recellularization of acellular ECM scaffolds with an appropriate cell source is increasingly being studied as a key component for vascular tissue engineering

applications.[2–4] In this study, we used adipose stem cells due to their high clinical potential and ease of harvesting.[2,4–6] Previously we have also discussed the immunomodulatory potential of these cells and many groups have looked into the potential of ADSCs to mitigate the inflammatory response in experimental disease models and tissue engineering applications.[7–10] The effect of seeding ADSCs into ECM scaffolds implanted sub-dermally into diabetic rats has already been investigated to study their immunomodulatory properties.[1] For this study, we decided to pre-differentiate the ADSCs into vascular cell lineages to see whether the same immunomodulatory effects would be observed in our vascular graft scaffolds in vivo. The previous chapter highlighted the benefits of PGG-treatment on inhibiting diabetes-related inflammation and degradation of our ECM scaffolds. Because ADSCs play an important role in our tissue engineering paradigm, our goal was to investigate the effect of seeding differentiated ADSCs and evaluate their ability to aid our tissue engineered vascular grafts in becoming diabetes-resistant. We also wanted to determine whether ADSCs could successfully pre-differentiate into the vascular cell lines of endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts (FB). This differentiative capability into vascular cell lineages has been mentioned in various papers.[4,11,12] Pre-differentiation allows us to guide these ADSCS into the desired cell types, which may aid in proper host cell infiltration with decreased migration of inflammatory cells. Treated with the appropriate growth factors of the correct concentrations, ADSC can be successfully differentiated into ECs, SMCs, and FBs. For endothelial cells, we treated the cells with VEGF, and IGF-1, for smooth muscle cells, we treated with TGF-β and BMP-4, and for

fibroblasts, we treated the cells with TGF-β. These specific growth factors were selected based on previous research on how to effectively induce ADSC differentiation into the vascular cell lineages. As seen in figure **5.1.**3, immunofluorescence staining for specific vascular cell markers showed that the ADSCs expressed the respective markers for ECs (CD31, vWF), SMCs (actin, SMH-Myosin, SM22), and FBs (actin, vimentin, Hsp47). This confirmed that differentiation had occurred and that the cells were ready for injection into the scaffolds. As observed in **Figure 5.1.4**, cell-seeding did not result in very much cell attachment and adhesion, however, recellularization was not the goal of this study. Rather, the objective was to inject all of the differentiated ASCs into the appropriate locations (ECs in the lumen, SMCs in the media, and FBs on the adventitia) to examine the effect of these cells on the scaffolds upon implantation.

After explanting the cell-seeded vascular grafts, histological evaluation with H&E and VVG Trichrome did not show any differences in host cell infiltration and elastin preservation (**Figure 5.1.4)**. This suggests that cell-seeding did not inhibit host cell infiltration, nor did it encourage more cell infiltration compared to the acellular grafts. Additionally, the original matrix integrity was not altered by the cell-seeding since the elastin fibers remained structurally intact. To determine whether cell-seeding affected formation of an endothelial lining and a smooth muscle layer, we looked for vWF and α-SMA (**Figure 5.1.5)**. Overall, it appears that cell-seeding does not have an effect on the host's ability to endothelialize the intima layer and lay down SMCs in the media in both diabetic and non-diabetic conditions. Interestingly, the acellular graft had a less dense and uniform SMC layer compared to the cell-seeded grafts. This, however, may not be of

significance since the acellular grafts in the study from chapter 4 showed SMC layer formation in the media with similar patterns as the cell-seeded grafts presented here.

Once it was confirmed that cell-seeding did affect the ECM quality, host cell infiltration, or formation of an endothelial layer and a smooth muscle layer, we proceeded to analyze the inflammatory response of the grafts. We noticed a slightly increased inflammatory response to the acellular grafts compared with the cell-seeded grafts implanted in the diabetic rats. When analyzed for CD8 (T-cells) and CD68 (macrophages) expression, 5.3% T-cells and 7.8% macrophage expression was observed in the acellular grafts and 3.6% T-cells and 3.6% CD68 expression was observed in the diabetic cell-seeded grafts. The non-diabetic cell-seeded grafts showed the lowest inflammatory cell infiltration, with 3.5% CD8 and 3.2% CD68 expression. Interestingly, the diabetic cell-seeded grafts showed the most CD163 expression, at 4.8% and the acellular had the lowest CD163 expression at 3.5%. Remarkably, none of the groups showed significant infiltration of iNOS. However, the cell-seeded groups did show slightly higher expression of iNOS (~1.5%) compared with the acellular graft (0.9%). This difference was considered negligible, and it was concluded that at 3 months, M1 macrophages did not play a role in the inflammatory response of these grafts. It is now increasingly recognized that ASCs exert their immunomodulatory effects by influencing the phenotype of macrophages and immunosuppressing T-cells.[1] ADSCs modulate the T-cell response from an inflammatory state to a quiescent, regenerative mode and balance overall Th1 and Th2.[10,13,14] The host innate immune system responds to implanted biomaterials by recruiting macrophages that amplify the inflammatory response and

consequently send signals to recruit T-cells.[15,16] However, we have observed that these ECM-based scaffolds are able to promote a switch in the macrophage population from a predominantly M1 phenotype to predominantly M2 macrophages. From our results, it appears that the cell-seeded grafts are able to promote more M2 macrophage expression compared to the acellular grafts and thereby reducing T-cell recruitment, further validating the immunomodulatory capabilities of the ADSCs. It is interesting that even after differentiating the ADSCs, they do not lose this immunomodulatory capability and are able to exert these effects on grafts implanted in diabetic conditions, which is extremely promising for future development of tissue engineered vascular scaffolds for diabetic patients.

As we learned in the previous chapter, PGG-treatment greatly aided in reducing the oxidative stress and AGE accumulation that is typically accelerated in diabetes. To determine whether the seeding of differentiated ADSCs would have a similar effect on the diabetic grafts, we looked for AGEs and NADPH oxidase 4, the same markers we tested for earlier. The diabetic grafts, although cell-seeded, still showed higher amounts of AGE accumulation and NADPH oxidase-4 compared to the non-diabetic grafts with cells and without cells. Therefore, it was concluded that cell-seeding, while aiding in inhibiting an inflammatory response, does not mediate the oxidative effects of diabetes.

5.5. Conclusion

From these studies we learned that ADSCs can be differentiated into endothelial cells, smooth muscle cells, and fibroblasts. Evaluation of the grafts seeded with the differentiated cells showed a reduced inflammatory response characterized by decreased

T-cell and overall macrophage migration but an increase in M2 macrophage presence. While these same results were observed in previously published work when non-differentiated ADSCs were injected into scaffolds, it was notable that we observed the same effects even after the cells had undergone differentiation. Cell-seeding did not, however, reduce AGE accumulation or oxidative stress in the grafts, indicating that it cannot replace the antioxidant properties of PGG. Therefore, it would be interesting to study the combined effects of PGG-treatment and differentiated ADSC cell-seeding to determine their ability to provide tissue engineered ECM scaffolds with even more resistance to diabetes.

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CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1. Conclusions

Patients with diabetes mellitus have an increased risk of developing cardiovascular diseases (CVD).[1] The development of CVD is initiated in the vascular system, where high levels of blood sugar binds to the tissues of blood vessels and triggers severe inflammation, stiffening, and degradation which progresses into atherosclerosis and eventually, arterial diseases such as coronary and peripheral artery disease.[2,3] Despite the extensive research and advancements in the treatment of CVD, there are still numerous amputations and vascular disease related deaths associated with diabetes. According to the AHA, about 500,000 coronary artery bypass graft procedures are performed every year in the US and about 12 million patients in the US have some form of peripheral artery disease; the American Diabetes Association estimates that 20-50% of these patients are diabetic.[4,5] Unfortunately, diabetic patients experience higher arterial disease-related mortality compared to non-diabetic patients.[6] Patients with diabetes have a high incidence of restenosis after receiving drug eluting stents,[7] and coronary artery bypass grafting occlusions are more common among diabetics versus non-diabetics at 1-year angiography.[8] Therefore, we identified this as a major need for vascular grafts for long-term stability and patency for diabetic patients. Tissue engineering provides immense potential for the replacement of these diseased tissues by regeneration and restoration of function and structure. It is also considered a patient-tailored approach, as it involves completely decellularized ECM based scaffolds and autologous cells.

However, the complications resulting from diabetes is a major hurdle for treating these patients. This tissue engineering application cannot be considered patient-tailored for diabetes-related revascularization procedures if cannot address the comorbidities associated with the patient. In addition to impairing the body's metabolic homeostasis, it also complicates the reparative and surgical methods used to treat the CVD. Our tissue engineering paradigm consists of 2 main components: completely biological scaffolds composed of ECM proteins and autologous stem cells, both of which are just as susceptible to AGE accumulation and glycoxidation as the native tissues. We have gained a better understanding of how tissue engineered constructs perform in diabetic environments, and we have concluded that the key to combatting diabetes-related alterations in our tissue engineering approach lies in the protection of the scaffolds.

Tissue engineered scaffolds have been proposed as an ideal source due to their biocompatibility, biodegradability, mechanical stability and similarity to native tissues, and natural ECM architechture.[9,10] Additionally, biologic scaffolds derived from allogeneic and xenogeneic sources can be eliminated of cells and immunogenic material while simultaneously retaining matrix integrity, mechanical properties, and key cell signaling molecules.[11,12] The primary motivation for using ECM based vascular scaffolds is to provide the optimal environment to encourage proper host cell infiltration as well as promote success in cell seeding prior to implantation. We believe that an ideal niche will be provided by the acellular ECM scaffolds. To do so, we have developed an effective decellularization procedure that completely remove cells and antigenic proteins while

preserving the natural 3D architecture and the major matrix components (specifically elastin).

Through these studies, we have shown that left untreated, diabetes will structurally alter the ECM scaffold thereby changing the vascular environment to pathological state. We demonstrated that diabetes results in a significantly higher inflammatory response by the host to the implanted scaffold compared to scaffolds implanted in non-diabetic hosts. Decreased patency, increased AGE accumulation and oxidative stress was also observed in the diabetic scaffolds. By treating the scaffolds with PGG, we inhibited the pathological structural modifications of the scaffold by matrix stabilization and mitigation of inflammation. The antioxidant and anti-inflammatory properties of PGG resisted glycoxidation and AGE accumulation thus rendering our tissue engineering vascular grafts "diabetes-resistant" to allow for constructive host cell infiltration, tissue integration, and functionality.

We have also shown that "pre-differentiated" ADSCs retained their immunomodulatory properties and inhibited inflammatory cells and shifted macrophage polarization from inflammatory to reparative. However, differentiated ADSC seeding onto the scaffolds did not prevent AGE accumulation and glycoxidation in the diabetic hosts like PGG, therefore, PGG-treatment is still necessary to provide the extra source of antioxidation and anti-inflammation. The end goal is now a synergistic system between PGG and pre-differentiated stem cells to produce a viable tissue engineered vascular graft for clinical translation of vascular tissue engineering for patients with diabetes.

6.2. Limitations and Challenges

Along the way, this research presented us with a plethora of limitations and challenges. The focus of this research has been on a narrow scope of diabetes, in which the effect of hyperglycemia on the vascular system was investigated. The small animal model utilized mimicked type I diabetes due to the destruction of pancreatic islets by STZ and subsequent insulin removal. Furthermore, the average blood glucose levels of the diabetic rats were relatively high (~600mg/dL), which is much higher than that seen in diabetic human patients. In order to further analyze the effects of diabetes on tissue engineering, use of animal models for type 2 diabetes would be necessary for *in vivo* studies. A major challenge that was presented during the animal studies was the survival rate of the rats post-surgery. Unlike the animal studies conducted before, which were subdermal implantations, these studies aimed at implanting the scaffolds as functional vascular grafts. This was a much more complicated and invasive procedure and many of the rats could not recover from the lengthy anesthesia exposure and faced difficulties in healing post-surgery. On average, we had a 40-50% survival rate for these surgeries, which resulted in the loss of several samples and experimental groups. For example, in chapter 5, we do not have an acellular scaffold in diabetes group due to the loss of animals after surgery. This was considered to be one limitation of using a small animal model for an invasive surgical procedure.

Achieving adequate cell seeding in the scaffold prior to implantation was a significant challenge. Due to limited time, we had to rely on static cell seeding with only overnight incubation. Based on the hematoxylin and eosin staining, there were very few

cells that adhered to the scaffold. Therefore, the impact of cell seeding may not have been entirely highlighted. Since the scaffold dimensions were so small, it was not possible to incorporate bioreactor conditioning to improve cell attachment after seeding. Another limitation of the small scaffold size was that after explanting, there was not enough tissue to also perform mechanical testing to determine how diabetes and implantation affected the mechanical properties of the scaffolds.

Finally, a major limitation was the length of surgical time. Due to the complexity of the surgery, implantation of one graft requires ~90 minutes, so in a single day, only 4 grafts could be implanted. For 25 animals, 7-10 days were needed to complete the surgeries, keeping the surgeon's and animal facility staff's time in consideration.

6.3. Recommendations for Future Work

6.3.1. Repeat Animal Studies with Larger Sample Size and Larger Animal Model

Due to the loss of animals during the surgical procedure, it would be beneficial to repeat the animal studies described in chapters 4 and 5 with more animals, to allow for a larger sample size for more extensive characterization. Ideally, with a 40-50% survival rate, at least 40 animals should be implanted with the grafts to allow for a sample size of 20-25. With a larger sample size, the explanted grafts could be divided into more characterization groups to include mechanical testing, protein and MMP analysis and degradation studies, as well as testing for specific AGE products such as CML and Pentosidine via IHC.

Eventually, these studies should move forward to incorporate larger animals such as rabbits or pigs. In doing so, we can evaluate the effect of diabetes on tissue

engineering in larger cardiovascular systems with higher translatability to human subjects. Furthermore, larger animals mean we could minimize the number of animals but have larger scaffold dimensions. This would still allow for more characterization and studies on the scaffolds using the same tests listed above. In the large animal studies, calcification should also be analyzed using Alizarin Red staining, and other osteogenic markers such as ALP, osteopontin, and osteocalcin using IHC. Since calcification did not occur in the rat model, it would be interesting to see if it would develop in a larger animal model of diabetes.

6.3.2. In vitro Analysis of Differentiated ADSCs

Additional studies on the differentiation capabilities of ADSCs would provide further insight into how they function when implanted *in vivo*. It would also be interesting to learn how the "pre-differentiated" ADSCs compare to native vascular cells. Furthermore, the cells should be grown in diabetic culture to compare the phenotypic changes that occur compared with non-diabetic conditions. This can be investigated with protein and gene analysis. This includes western blotting, RNA isolation and RT-PCR. Proteins to look for should include α-SMA, SMH-myosin for SMCs, CD31 and vWF for ECs, Vimentin and Hsp47 for fibroblasts.

6.3.3. Mechanical Testing of Explanted Grafts

Explant analysis should also include examination of mechanical properties. Mechanical testing should include biaxial tensile tests for stress-strain characteristics and thermal denaturation temperature from DSC. This will provide additional insight into the

effect of diabetes on the scaffolds and whether PGG-treatment and cell seeding protect the scaffolds from mechanical alterations.

6.3.4. In vivo analysis with Combined Differentiated ADSC Cell-Seeding and PGG-Treatment

To evaluate whether the anti-inflammatory properties of the differentiated stem cells and anti-oxidant would work in combination to further render the ECM scaffolds diabetes resistant, an *in vivo* study of PGG-treated and cell-seeded scaffolds should be planned. There should be 8 experimental groups as follows:

- Diabetic, PGG-treated, cell-seeded
- Diabetic, PGG-treated, acellular,
- Diabetic, non-treated, cell-seeded
- Diabetic, non-treated, acellular
- Non-diabetic, PGG-treated, cell-seeded
- Non-diabetic, PGG-treated, acellular
- Non-diabetic, non-treated, cell-seeded
- Non-diabetic, non-treated, acellular

There should be at least 5 rats per group so a total of 50 animals should be ordered to accommodate for any losses. The grafts will be implanted as described in the studies in chapters 4 and 5 and diabetes will be induced using STZ administration. Explant analysis should examine mechanical properties, host cell infiltration, AGE accumulation, calcification, matrix integrity, and inflammation.

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